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Pathogenesis of Post Surgical Adhesions and Prevention using a Novel Fibrin Sealant

A thesis submitted for the degree of Doctor of Philosophy

by

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Abstract

Post surgical adhesions (PSAs) are an inevitable outcome of surgery and their presence leads to pathogeneses and significant economic impact.

The studies within this thesis utilised standard and reproducible abrasion models, in rabbits, pigs and rats, to investigate the formation and maturation of PSAs with strict quantitative analyses. These studies have shown that the development of PSAs is a series of complex, multi-factorial processes. PSA development can be classified into two stages: (i) PSA modelling occurring up to/including 16 hours post injury characterised by the inflammatory response and fibrin deposition and maturation; and (ii) PSA remodelling occurring from 16 hours onwards and characterised by tissue repair, collagen deposition and maturation and chemical mediation by TGF-β.

Treatment with Vivostat™ System Derived (novel) Fibrin Sealant significantly reduced the formation of PSAs with mean PSA reduction of: 80% for the rabbit uterine horn abrasion model, from 3 separate studies; 83% for the pig stomach/colon/caecum abrasion model, from 2 separate studies; 80% for the rat caecum abrasion model. This is significantly better than other fibrin sealants investigated in this thesis.

PSA prevention with novel fibrin sealant demonstrated a similar pattern to PSA development, with two stages of development evident: (i) tissue generation modelling occurring up to/including 16 hours post injury characterised by the inflammatory response and fibrin deposition and maturation; and (ii) tissue generation remodelling occurring from 16 hours onwards and characterised by tissue repair, collagen deposition and maturation and chemical mediation by TGF-β. However the extent and subsequent time taken for these changes to occur was significantly reduced.

The prevention of PSAs and alterations of wound healing by novel fibrin sealant is most probably due to the sealant acting as a haemostat, as well as a physical barrier. Thus preventing fibrinous and subsequent fibrous PSA formation.

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Table of Abbreviations

CMC	Carboxymethylcellulose
Conc^{n}	Concentration
CV%	Percentage coefficient of variation
GM-CSF	Granulocyte macrophage colony stimulating factor
HA	Hyluronic acid
IFN	Interferon
IL	Interleukin
IMS	Industrial methylated spirit
LPS	Lipopolysaccharide
M-CSF	Macrophage colony stimulating factor
Novel fibrin sealant	Vivostat™ system derived fibrin sealant
NSAIDS	Non steroidal anti-inflammatory drugs
PA	Plasminogen activator
PAA	Plasminogen activator activity
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PMNLs	Polymorphonuclear leukocytes
PSA(s)	Post surgical adhesion(s)
PTAH	Phosphotungstic Acid Haematoxylin
PTG	Peritoneal tissue generation
RBCs	Red blood cells
SD	Standard deviation
SEM	Standard error of the mean
TG	Tissue generation
TGF	Transforming growth factor
TNF	Tumour necrosis factor
tPA	Tissue plasminogen activator
TTG	Total tissue generation
UCF	Unbiased counting frame
UTG	Uterine horn tissue generation
WBCs	White blood cells

1.0 Introduction

1.1 Post Surgical Adhesions (PSAs)

Adhesions are linkages which join together organs which would normally be separate and freely moveable. Their presence causes an upset in normal physiology resulting in potential morbidity and mortality. They are highly differentiated tissues, consisting of connective tissue proteins and cellular elements, with exact composition varying throughout their development. Dependent upon their developmental stage, adhesions can be defined as:

i. fibrinous - primary transient adhesions, consisting primarily of fibrin, together with cellular components
ii. fibrous - secondary permanent adhesions, consisting primarily of vascular collagen, with scattering of cells.

Adhesions are also defined according to their origin. They are generally classified as:

i. inherited or congenital adhesions - these are present from birth. There are two schools of thought as to the origin of these adhesions:
   a. anomalies due to the failure of the gut to rotate and descend or other developmental anomalies
   b. pre-natal inflammatory conditions within the peritoneal cavity
ii. acquired adhesions - these may be further divided into:
   a. inflammatory adhesions - resulting from inflammatory conditions such as pelvic inflammatory disease, endometriosis, acute cholecystitis, acute diverticulitis and peritonitis
   b. post surgical adhesions (PSAs).

1.1.1 History

Causes of adhesions and associated problems have varied over the last two centuries and so in turn has the clinical interest in them. Until the advent of reliable and safe anaesthesia and aseptic surgical techniques, surgical procedures were risky and rare, and

hence so were surgically associated sequelae. Consequently only inflammatory and congenital adhesions were discovered, usually limited to unusual findings at post mortem (Hodgkin, 1836). However by the end of the last century surgical treatments for pathogeneses were becoming more common and were soon followed by reports of PSAs usually fatal (Bryant, 1872; Battle 1883). Since this time reports of and interest in PSAs have gone hand in hand with the increased use of surgical procedures.

Although surgical techniques have been maximised over many decades to increase their efficiency and effectiveness and decrease associated problems it is doubtful that surgical sequelae will ever be totally eradicated. The formation of adhesions, following many types of surgical procedures is almost inevitable and presents major problems for both surgeons and patients alike. PSAs are associated with morbidity and potential mortality, resulting, in some cases, in: failed surgical therapy; the need for re-operation or conservative treatment; complication of further surgical procedures; increased workload; and a large economic burden in today's climate of limited resources (DiZerega, 1994; DeCherney & DiZerega, 1997; Holmdahl & Risberg, 1997).

1.1.2 Associated Pathogeneses

It is agreed that the main problems arising from PSAs in the peritoneum are intestinal obstruction, infertility, pain, and complications with/leading to re-operation within the peritoneal cavity. However since any invasive surgical procedure has the potential to induce PSA formation, reports of PSAs ensuing from pericardial (Youmans *et al.*, 1968; Cliff *et al.*, 1973; Milgalter *et al.*, 1985; Mitchell *et al.*, 1994), pleural (Youmans *et al.*, 1968; Kaiser *et al.*, 1979) and orthopaedic (Bucknall & Ellis, 1984; Gelberman & Manske, 1985; Hagberg *et al.*, 1991; Frykman *et al.*, 1993) practices are also common. Understandably, pericardial adhesions have great significance due to the unique function of the heart and the increased number of cardiac surgical therapies carried out in a society where heart disease is a major cause of morbidity and mortality in adults.

1.1.3 Incidence

Incidence of the different types of adhesions have shown variation over the last century, corresponding with the trend of surgical treatment for disease. Information regarding the incidence of adhesions has been limited to post mortem reports, observations made during surgical procedures and in more recent years "second look" studies, usually laparoscopic in nature (Bulletti *et al.*, 1996; Fernandez *et al.*, 1996; Edelstam *et al.*, 1998).

1.1.3.1 Overall

An autopsy study carried out in 1973 by Weibel and Majno found the incidence of adhesions to be 44.5% from 752 subjects (336 women and 416 men). Their data was comparable to the 44.9% adhesion incidence reported by Benzer *et al.*, (1963) and greater than the 31.1% found by Diebold in 1930. Weibel and Majno (1973) hypothesised that the increase in incidence seen between 1930 and 1960/1970's corresponded to increased surgical practice over this period.

1.1.3.2 Adhesion Types

In 1952 Nemir *et al.*, determined the cause of adhesions causing bowel obstruction (adhesional obstruction) to be post surgical in 79% of cases and congenital in the remaining 21%. Similar results were found by Perry *et al.*, (1955) with PSAs accounting for 79.4%, inflammatory adhesions 17.8% and congenital adhesions 2.8% of adhesional bowel obstructions. A status quo appears to have occurred from the 1950's to 1970's, with Weibel and Majno's study in 1973 reporting that of adhesions found, 67% were post surgical and 28% inflammatory/congenital in nature.

However in 1990 Menzies and Ellis reported the incidence of adhesions following major abdominal surgery to be 93%, with 9.6% occurrence for inflammatory adhesions and 0.9% for congenital adhesions. Hence over the past twenty years there is a pattern for increased incidence of PSAs, with a decline in inflammatory/congenital adhesions. This has also been reported by Duron *et al.*, (1993), who also demonstrated a 93% presence

of PSAs following abdominal surgery.

This pattern for increased PSAs and decreased spontaneous adhesions has occurred in developed countries only. A study on adhesional obstruction in Nigeria by Chiedozi *et al.*, (1980) reported there was almost equal division between post-traumatic and post-inflammatory adhesions (54.3% and 45.7% respectively). This pattern can be accounted for by the lower number of laparotomies performed in developing countries compared to the western world and also to the relatively high incidences of typhoid, infectious hepatitis and other causes of primary peritoneal inflammation in the African patient population.

Interestingly there also appears to be a correlation between the type of adhesion and location within the abdominal cavity (Perry *et al.*, 1955): adhesion involving the small and large bowel (mixed) and large bowel only were predominantly inflammatory (30.5%), then congenital (22.3%) and only a small portion post operative (6.8%). However the small bowel is much more susceptible to post surgical adhesional obstruction (88.4% of cases) compared to large (8.0%) and mixed (3.6%) obstructions.

1.1.3.3 Previous Operation(s)

Weibel and Majno (1973) showed that the incidence of adhesions was dependent upon the type of previous operation, with incidence increasing after minor (51%), major (72%) and multiple (93%) operations respectively. In contrast Luijendijk *et al.*, (1996) found no difference between one minor and major operation however the number of adhesions was significantly lower in patients with a history of one operation compared to multiple operations. In addition the number of adhesions was significantly greater in patients with previous PSAs and those with surgical complications, such as abscesses, haematomas and intestinal leakages (Luijendijk *et al.*, 1996).

1.1.3.4 Age

Several studies have detailed the effect of patient age on the occurrence of adhesions.

Weibel and Majno noted that spontaneous adhesions occurred more frequently after the age of 60, although age did not exert an effect on the incidence of PSAs. Studies by Ray *et al.*, (1993; 1998) detailing hospitalisation rates for adhesiolysis both show a bimodal distribution with age, with highest rates seen in adults between 26 and 50 years old, followed by adults 65 years and older in the 1988 study and reversal of these two groups in 1994. However it should be remembered that studies by Ray *et al.*, (1993; 1998) only provide details on problematic adhesions rather than adhesions *per se*.

1.1.3.5 Sex

Weibel and Majno (1973) demonstrated that women have approximately 4% more PSAs and spontaneous adhesions compared to men. This observation is supported by the adhesiolysis hospitalisation rate studies by Ray *et al.*, (1993; 1998) who reported rates six and four times greater for women compared to men respectively.

1.1.3.6 Obesity and Height

The development of PSAs is greater in short and obese patients, both women and men (although less pronounced in men) compared to obese patients of average height or taller or thin patients (Weibel and Majno, 1973). This study also recorded that obese women had greater incidences of spontaneous adhesions compared to thin women, however this observation may be better attributed to sex as obesity showed no effect on the incidence of spontaneous adhesions in men.

1.1.3.7 Adhesional Intestinal Obstruction

Postoperative bowel obstruction is a relatively rare but serious surgical complication. The incidence of early postoperative bowel obstruction (adhesional obstruction) has been reported to be 0.5% (Menzies & Ellis, 1990) and 0.69% (Stewart *et al.*, 1987) of laparotomy cases, with this figure rising to 1% within one year of surgery (Menzies & Ellis, 1990). Higher incidences of adhesional bowel obstruction following laparotomy have been reported in neonates (2.2% (Festen, 1982) and 8.3% (Wilkins & Spitz, 1986)). The higher figures demonstrated in neonatal surgery is probably due the need for

increased precision, greater difficulty and hence increased operating time, with this hypothesis supported by more traumatic neonatal surgical procedures carrying a greater risk of adhesional obstruction development (Wilkins & Spitz, 1986). However the risk of developing small bowel obstruction has been shown to be not significantly higher in neonates compared to infants and older children (Festen, 1982).

In the western world, strangulated hernias were the most common causative factor for bowel obstruction during the first half of this century, however since the 1950s adhesions have become the primary cause (Table 1.1).

Author(s)	Publication Year	Survey Year(s)	Country	% Causing Bowel Obstruction	
				Adhesions	Hernias
Souttar	1925	1920-1925	Britain	11.2	46.0
Vick	1932	1925-1930	Britain	7.3	47.4
Nemir *et al.*,	1952	1940-1950	USA	39.7	25.1
Perry *et al.*,	1955	1942-1953	USA	31.0	10.2
Playforth *et al.*,	1970	-	USA	54.0	23.0
Stewardson *et al.*,	1978	1967-1976	USA	66.0	24.0
Bizer *et al.*,	1981	1968-1978	USA	74.0	8.1
Menzies & Ellis	1990	1984-1987	Britain	51.0	-
Stricker *et al.*,	1994	1988-1991	USA	59.0	6.0

Table 1.1: Percentages of bowel obstructions caused by adhesions and strangulated hernias in the western world.

The pattern demonstrated is hardly surprising considering how surgical practices have changed throughout this century, with increased number of surgical procedures and early elective treatment of hernias contributing. The effect of abdominal surgery on bowel obstructions is also highlighted by data from less developed countries, who perform laparotomies to a much lesser extent (Table 1.2) and reflect the pattern seen during the first half of this century in the western world.

Author(s)	Publication Year	Survey Year(s)	Country	% Causing Bowel Obstruction	
				Adhesions	Hernias
McAdam	1961	1958-1960	Uganda	4.0	74.8
Cole	1965	1957-1962	Nigeria	10.6	37.6
Brooks & Bulter	1966	1952-1963	Jamaica	23.2	25.0
Chiedozi *et al.*,	1980	1973-1978	Nigeria	10.8	65.0

Table 1.2: Percentages of bowel obstructions caused by adhesions and strangulated hernias in the developing countries.

The small bowel is one of the most predominant areas for the formation of adhesions. Distribution of PSAs has been shown to involve the omentum (68%), small bowel (67%), abdominal wall (45%), colon (41%), liver (34%), female reproductive organs (23%), stomach (20%), retroperitoneum (14%) and spleen (9%) (Luijendijk *et al.*, 1996). The high involvement incidence of PSAs associated with the small bowel have also been reported by Perry *et al.*, (1955) (88.4%) and Menzies (1992)(60-70%). This predilection is probably associated with the location of the small bowel in the peritoneal cavity, both with regard to operative techniques and laparotomy wound.

Perry *et al.*, (1955) reported that operations on the large bowel, appendectomies and intestinal obstruction were the most common preceding surgical procedures. This was confirmed by Menzies & Ellis (1990) who found that of the previous operations performed 25% concerned the colon and rectum, 15% were appendectomies, 14% gynaecological operation and 9% total colectomies. Since these are some of the most common surgical interventions performed, problems of PSAs are magnified in a "catch 22" situation.

Development of post operative adhesional bowel obstruction can occur many years following surgery. Menzies & Ellis (1990) demonstrated that 21% of obstruction occurred within one month of surgery, 18% from 1 month to 1 year, 21% between 1 and 5 years, 6% from 5 to 10 years and 21% after 10 years of the surgical procedure. Similar results have been reported by many authors, for example Stricker *et al.*, (1994) found the median period of time from previous operation to intestinal obstruction to be two years,

whilst Brightwell *et al.*, (1977) found that half of patients developed bowel obstruction within 4 months. However it is well known that the clinical manifestation of bowel obstruction may occur many years following the original surgical procedure (Brightwell *et al.*, 1977; Bevan, 1984; Stricker *et al.*, 1994).

Overall mortality rates related to adhesional bowel obstruction have been reported between 3% (Stewardson *et al.*, 1978) to 17.8% for early postoperative small bowel obstruction (Stewart *et al.*, 1987) (Table 1.3) .However they can range between 0 and 37% depending upon the type of adhesion, patient age, surgical complications, duration prior to treatment and the type of treatment.

Author(s)	Year	Country	Mortality Rate (%)
Vicks	1932	Britain	32.9
Nemir	1952	USA	7.5
Perry *et al.*,	1955	USA	6.0
Stewardson *et al.*,	1978	USA	3.0
Bevan	1984	Britain	6.7
Stewart *et al.*,	1987	USA	17.8
McAdam	1961	Uganda	12.5
Cole	1965	Nigeria	~13.0

Table 1.3: Mortality rates for adhesional bowel obstruction.

Following diagnosis, type and timing of treatment for intestinal obstruction still remains a delicate matter. Conservative treatment appears to give an advantage in both morbidity and mortality when effective (Perry *et al.*, 1955), avoiding surgical consequences such as the formation of further adhesions. However non surgical intervention can be disadvantageous if it proves to be ineffective. The longer the obstruction remains the greater the chance of associated pathogeneses, such as intraperitoneal abscesses, strangulation, gangrene and perforation, which is related to increased mortality (Perry *et al.*, 1995; Ellis, 1997).

Even with effective treatment the risk of adhesion reoccurrence is still present. Many experimental studies have stated high incidences of adhesion reoccurrence following division of existing adhesions (79% Menzies & Ellis, 1989; 100% Gervin *et al.*, 1973). Clinically the probability of adhesion reformation has been stated as 12.5% (Bizer *et al.*,

1986) for conservative treatment and from 13 to 32% following surgical division (Bizer *et al.*, 1986; Brightwell NL *et al.*, 1977).

1.1.3.8 Gynaecological Problems

Previous gynaecological pathology has been shown to play a significant role in adhesional bowel obstruction. Stricker *et al.*, (1994) found that 48% of patients with intestinal obstruction had previous gynaecological or obstetric problems or associated surgery respectively, with hysterectomy being the most common previous operation. Due to this association gynaecologists manage more than 20% of all female patients with intestinal obstruction.

Adhesions are a significant causative agent of female infertility, with 15 - 20% attributed to adhesion presence (Soules *et al.*,1982; Hull *et al.*, 1985). A study by Rapkin (1986) found that 39% of infertile patients exhibited pelvic adhesions as the only abnormal finding. In addition many studies have demonstrated an inverse relationship between pregnancy rate and pelvic adhesions (Rock *et al.*, 1978; Caspi *et al.*, 1979; Caspi & Halperin, 1981). The importance of the effect of adhesions on gynaecological conditions is demonstrated by the fact that the female reproductive system was the primary site of adhesiolysis procedures, accounting for 47% of all adhesiolysis hospitalisations (Ray *et al.*, 1998).

Chronic pelvic pain is one of the most common gynaecological complaints, yet its actual pathology still remains undefined and perplexing. It is believed by many that there is an association between pelvic pain and adhesions, associated with previous surgery or pelvic inflammatory disease. Kersch *et al.*, (1984) demonstrated that adhesions were the most common pathology associated with abnormal pelvic organs and chronic pain, however not all pelvic adhesions caused pain. They hypothesised that the difference between painful and pain-free pelvic adhesions was dependent upon their restriction on organ mobility and expansion, however they state that this structural mechanical explanation is not the whole answer. However, Rapkin (1986) questions whether pelvic adhesions do in fact play such an important role in the pathogenesis of chronic pelvic

pain.

1.1.3.9 Laparotomy verses Laparoscopy

During the last decade there has been a trend for increased use of laparoscopy for appropriate abdominal procedures. This endoscopic procedure carries the obvious benefits of a less invasive technique, with a smaller incision wound, potentially reduced operative time, reduced recovery time and hence reduced patient care cost.

Although laparoscopy does induce PSA formation there have been many reports of reduced incidences compared to the open method of laparotomy, as long as meticulous surgical standards are employed (Luciano, 1990; Moore *et al.*, 1995; Bulletti *et al.*, 1996; Reissman *et al.*, 1996; Tittel *et al.*, 1996; Tulandi, 1997; Chen *et al.*, 1998). However reports by Filmar *et al.*, (1987) and Ellis (1997) are contradictory, stating that laparoscopy and laparotomy carry the same risk of inducing intra-abdominal adhesions. Support for these studies come from Ray *et al.*, (1998) who reported that the increased use of laparoscopy between 1988 and 1994 does not appear to be associated with a reduction in adhesiolysis hospitalisation rate.

The use of laparoscopy for surgical procedures will undoubtedly continue to become more popular due to increased patient comfort and decreased cost and workload to hospital staff. However the benefits of this technique with regard to PSA reduction is debatable.

1.1.4 Benefits

It is generally agreed that the formation of adhesions following an inflammatory or surgical insult is almost inevitable. Hence the formation of adhesions following tissue injury is a normal natural phenomena and it could be argued that adhesions are therefore beneficial.

The formation of a fibrin coat over injured tissue has obvious protective effects to the

underlying exposed tissue. However it has also been suggested that adhesions themselves are advantageous. Ellis (1962) hypothesised that the formation of adhesions was to provide damaged/ischaemic tissue with blood supply, calling adhesions "vascular grafts", preventing necrosis, gangrene or perforation. Similar views have been stated by Myllarniemi & Karppinen (1968) who quoted that if adhesion formation is prevented recovery may be hampered, creating conditions favouring infection and leading to complications such as gangrene and necrosis. In an early study by Deaver, (1923) limiting of infection by adhesion formation ("walling-off" process) was reported, with adhesions also providing fine strands and ladders along which phagocytic cells could travel to the source of infection in order to combat it.

Support for the hypothesis that PSAs are valuable comes from:

i. ischaemia promoting adhesion development (Ellis, 1962; Myllarniemi & Karppinen, 1968; Dunn & Mohler, 1990)
ii. rapid angiogenesis associated with adhesions (Myllarniemi & Karppinen, 1968)
iii. venous obstruction inducing adhesion formation (Belzer, 1967).

However healing of defects which do not induce adhesion formation are not associated with pathogenic conditions. Hence it appears that although benign adhesions provide adhered tissues with some benefits (protection of underlying tissues from mechanical damage and vascular supply) their potential pathogeneses far outweigh their advantages, a view which is held world wide by most surgeons.

1.1.5 Economic/Workload Impact

In addition to pathogenesis, PSAs impose a significant economic and workload burden in today's climate of limited resources.

In 1988 in the United States there were 281,982 hospitalisations during which adhesiolysis was performed, resulting in an estimated $1,179.9 million in expenditures, excluding outpatient care or the cost of failed surgical therapy due to adhesion development (Ray *et al.*, 1993). A follow up study by Ray *et al.*, (1998) reported a similar number (303,836) of hospitalisations for adhesiolysis in 1994, with an

estimated cost of $1.3 billion. Although hospitalisation rates were relatively constant between 1988 and 1994, comparative costs decreased between 1988 and 1994. This was related to a decrease in the average length of hospitalisation, a result of managed health care and less invasive adhesiolysis techniques (Ray *et al.*, 1998). Although adhesiolysis only accounted for approximately 1% of all hospitalisations (Ray *et al.*, 1998) they are a substantial drain on resources.

Since adhesions are now the most common cause of bowel obstruction in the western world (Nemir *et al.*, 1952; Perry *et al.*, 1955; Playforth *et al.*, 1970; Stewardson *et al.*, 1978; Bizer *et al.*, 1981; Menzies & Ellis, 1990; Stricker *et al.*, 1994) the economic and workload impact is self evident. A survey by Scott-Coombes *et al.*, (1993) showed that, in the United Kingdom, approximately 3600-4800 laparotomies per year are performed for adhesional bowel obstruction, with 8400 to 9600 admissions and an average hospital stay in excess of 15 days (McEntree *et al.*, 1987). In addition an average of 3600 laparotomies per year for non-adhesion related diseases are complicated by the presence of adhesions.

Similar statistics have been found in other parts of Europe. Holmdahl & Risberg (1997) reported an overall incidence of laparotomy for adhesional bowel obstruction to be 4%, with again twice as many admissions. This equates to 1900 laparotomies performed each year for this type of bowel obstruction, with an estimated cost of US$4.1 million. If the statistics included pain, dysfunction and infertility then an estimated US$11 million per year was spent on treatment of adhesion-related pathogeneses.

1.1.6 Formation

1.1.6.1 Peritoneal Mesothelium

The main functions of the peritoneum are to:

i. Allow friction free movement of abdominal organs
ii. "Wall off" infection within the abdominal cavity
iii. Act as a reservoir of fat (omentum)

These functions of the peritoneum are related to its structure.

The surface of the peritoneum is a single layer of loosely connected, flat, highly differentiated mesothelial cells, which possess a varying number of microvilli on their free surface. Due to the mesothelial cells being very poorly interconnected, the peritoneal surface is very delicate and susceptible to damage. Hence very minimal mobilisation or damage to the peritoneum causes uplifting of many mesothelial cells, leaving the surface denuded. Mesothelial cells rest on a basement membrane, which in turn rests on loose connective tissue. Connective tissue consists of collagen and reticular fibres and cellular elements - fibroblasts, lymphocytes, macrophages, adipocytes, plasma cells and mast cells (Johnson & Whitting, 1962; Baradi & Hope, 1964; Eskeland, 1966; Drollette & Badaway, 1992; DiZerega, 1997).

Pleural and pericardial membranes are the only other membranes with a similar composition, and healing and adhesion development associated with these membranes probably progresses in a similar manner to the peritoneum.

1.1.6.2 Peritoneal Fluid

This extracellular fluid, which bathes the organs of the peritoneum, is a plasma exudate and has a similar composition:

i. Plasma proteins, including large amounts of fibrinogen (DiZerega, 1997); and
ii. Cellular elements - macrophages, monocytes, lymphocytes, eosinophils, mast cells but no mesothelial cells (Eskeland, 1966.1; Eskeland, 1966.2; Raftery, 1973.L).

Peritoneal fluid has been shown to undergo both volumetric and component changes in response to surgery:

i. Increased volume and plasma protein components (DiZerega, 1997).
ii. Increased cell numbers
    - Shimanuki *et al.*, (1986) demonstrated that 6 hours after abrasion of the peritoneum, peritoneal fluid comprised of 87.5% polymorphonuclear cells, with Eskeland (Eskeland, 1966.1; Eskeland, 1966.2) observing that

polymorphonuclear granulocytes were present during the first three days following injury.

- 3 days following abrasion total cell number in peritoneal fluid increased with large mononuclear cells making up 97.6% (Shimanuki *et al.*, 1986).
- Reabrasion was followed by less leukocytes in the peritoneal fluid compared to the initial abrasion, however the percentage of macrophages was higher 24 hours following reabrasion (Shimanuki *et al.*, 1986).

1.1.6.3 Adhesion Free Healing

Interest in the healing of wounds has been considered ever since the advent of medicine. For many years the healing of all wounds was considered to be similar, for example, peritoneal wounds were thought to heal in the same manner as skin wounds, with gradual re-epithelisation from the wound boundary. However, in the early decades of this century, theories contradicting this hypothesis were purposed by Hertzler (1919) and Lewis (1923) who both suggested that re-epithelisation of peritoneal wounds occurred from subjacent tissues/cells. Although nowadays there are contradictory ideas concerning the origin of new mesothelial cells, it is agreed that mesothelial wounds (peritoneal, pleural and pericardial cavities) do differ in their healing mechanisms compared to skin and other defects.

The two main observations of interest regarding the healing of peritoneal wounds are:

i. The rapidity of healing of peritoneal wounds, with a continuous layer of cells covering the wound surface within 8 days following injury (Ryan *et al.*, 1973; Johnson & Whitting, 1962; Ellis *et al.*, 1965; Hubbard *et al.*, 1967; Raftery, 1973.L; Eskeland, 1966.1; Eskeland, 1966.2)
ii. Healing of small and large defects occurs within the same time period (Ellis *et al.*, 1965; Eskeland, 1966.1; Eskeland, 1966.2; Hubbard *et al.*, 1967).

1.1.6.3.1 Macroscopic Appearance

In the first 2 days following injury peritoneal wounds appear to have a glistening but

uneven haemorrhagic surface (Williams, 1955; Ellis *et al.*, 1965; Eskeland, 1966.1; Hubbard *et al.*, 1967), and by 3 to 4 days the wound surface is still red, velvety (Eskeland, 1966.1) and glistening (Williams, 1955). The difference between the wound surface and the surrounding peritoneum progressively decreases over time, with the wound surface becoming gradually more pale, smooth and shiny (Eskeland, 1966.1; Hubbard *et al.*, 1967). By 8 days the whole wound surface is smooth and glistening though still slightly more red than the surrounding peritoneum and at 14 days only a shallow depression of greyish white colour indicates the location of the wound (Eskeland, 1966.1).

1.1.6.3.2 Structural Changes

Overall there is agreement as to the structural changes which occur during adhesion-free healing of peritoneal wounds. Fibrin formation at the site of injury occurs rapidly (Figure 1.1) with fibrin strands present at 1 hour following injury (Eskeland, 1966; Eskeland, 1966). Fibrin strands form networks covering the wound surface during the first 2 to 3 days, after which fibrin absorption is initiated (Johnson & Whitting, 1962; Raftery, 1973.E). By 4 days post injury there is little or no fibrin in the wound exudate (Eskeland, 1966.1; Raftery, 1973.L; Raftery, 1973.E). Collagen fibres are observed at 5 days (Ryan *et al.*, 1973; Raftery, 1973.L), with thick collagen bundles seen in the base of the wound at 8 days and a thin layer of fibrous tissue covered by a continuous layer of mesothelium occupying the site of the original defect (Raftery, 1973.L). Little further change is seen thereafter, although the fibrous tissue appears to contract and reduce the size of the scar considerably (Ellis *et al.*, 1965)(Figure 1.2).

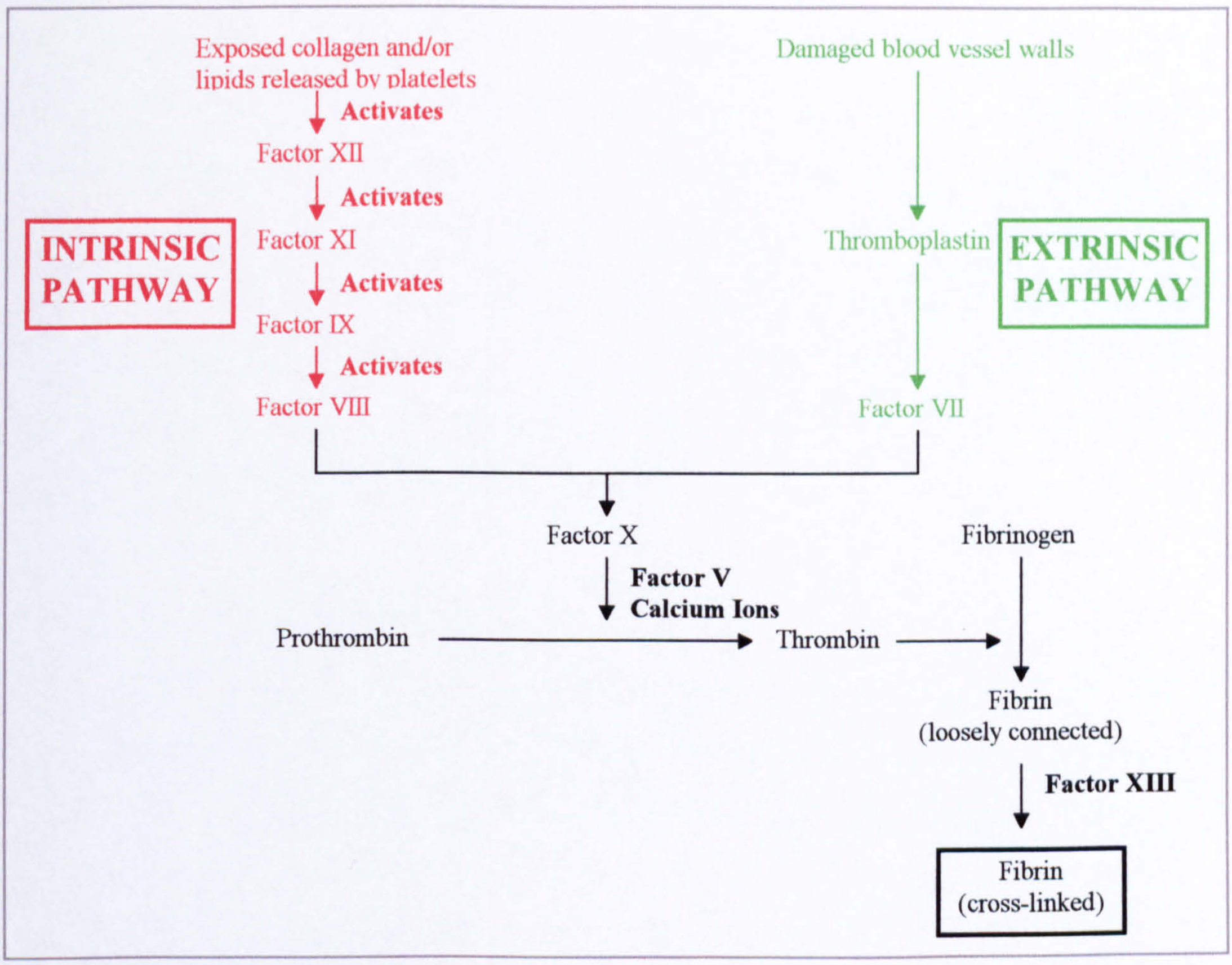


Figure 1.1: Fibrinogenesis pathway.

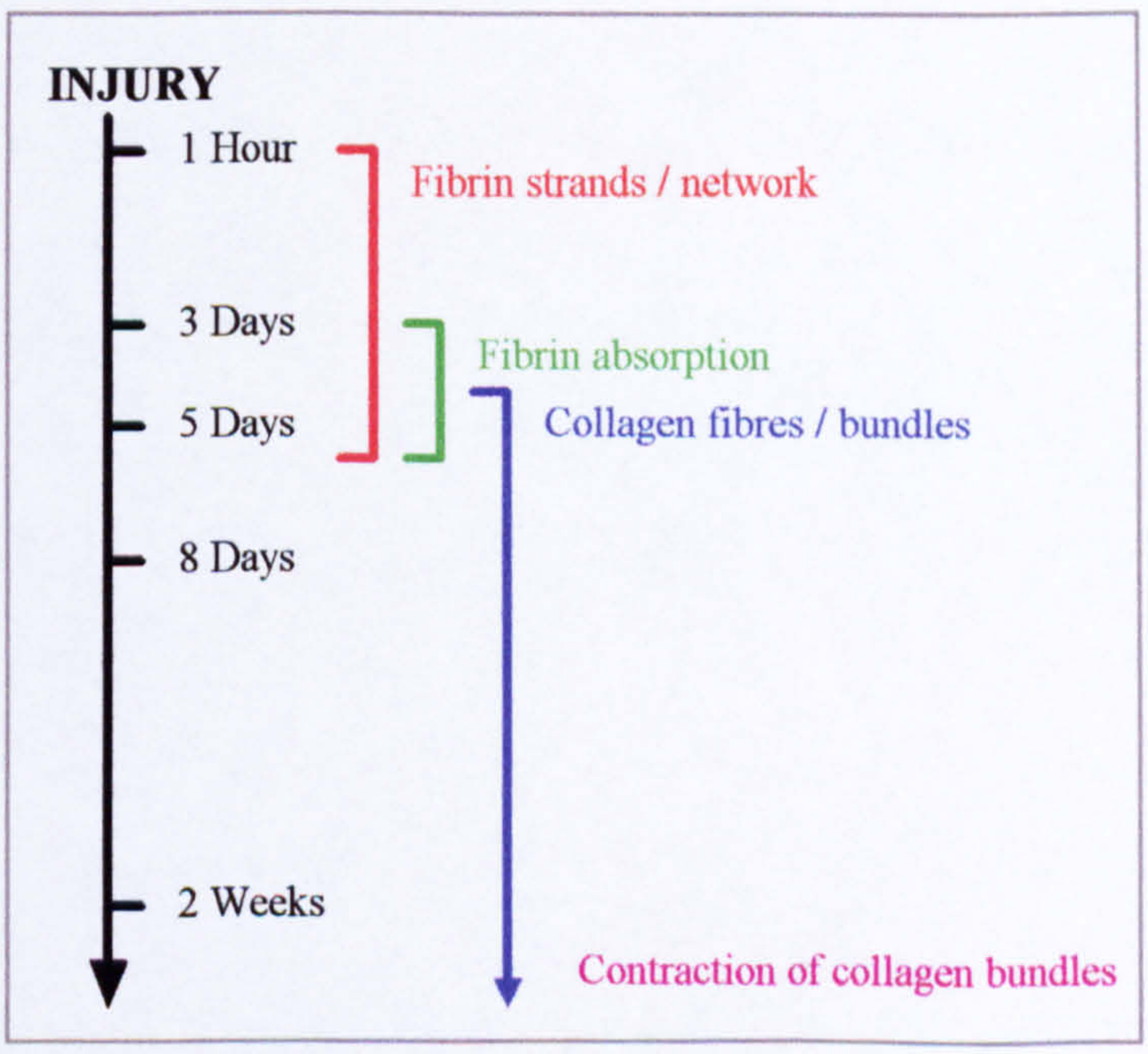


Figure 1.2: Structural changes occurring during adhesion-free wound healing.

In addition to connective tissue formation, the development of new blood vessels is essential for successful wound healing. Angiogenesis is a complex process involving the interplay between vascular and non vascular cells, soluble mediators and extracellular

matrix components. The process is the result of a cascade of overlapping steps: (i) degradation of basement membrane, (ii) migration of endothelial cells, (iii) proliferation of endothelial cells, and (iv) capillary tube formation and maturation. Macrophages play important roles in angiogenesis, through the release of both positive and negative chemical regulators (Appendix I Table 1.1) but mast cells, neutrophils and lymphocytes have also been implicated. Angiogenesis begins during the inflammatory stages of wound healing and new blood vessel formation has been demonstrated in peritoneal wounds after 4 to 6 days (Johnson & Whitting, 1962).

1.1.6.3.3 Cellular Involvement

Cellular actions and interactions play essential roles in normal and pathological wound healing (Appendix I Table 1.2). Cellular involvement can be classified as having three main stages: (i) haemostasis; (ii) inflammatory response; (iii) tissue repair. These will be discussed in turn.

(i) Haemostasis

Immediately following tissue injury, platelets (and some blood cells) exude from the damaged tissue. These platelets adhere to exposed collagen present at the wound site, and secrete granules containing serotonin, adrenaline, adenosine diphosphate (ADP) and several clotting factors. Serotonin and adrenaline cause blood vessels to contract, ADP results in the platelets becoming more adherent, leading to the formation of a platelet plug, and the clotting factors result in the formation of a fibrin clot. Hence the process of haemostasis is achieved (Figure 1.3).

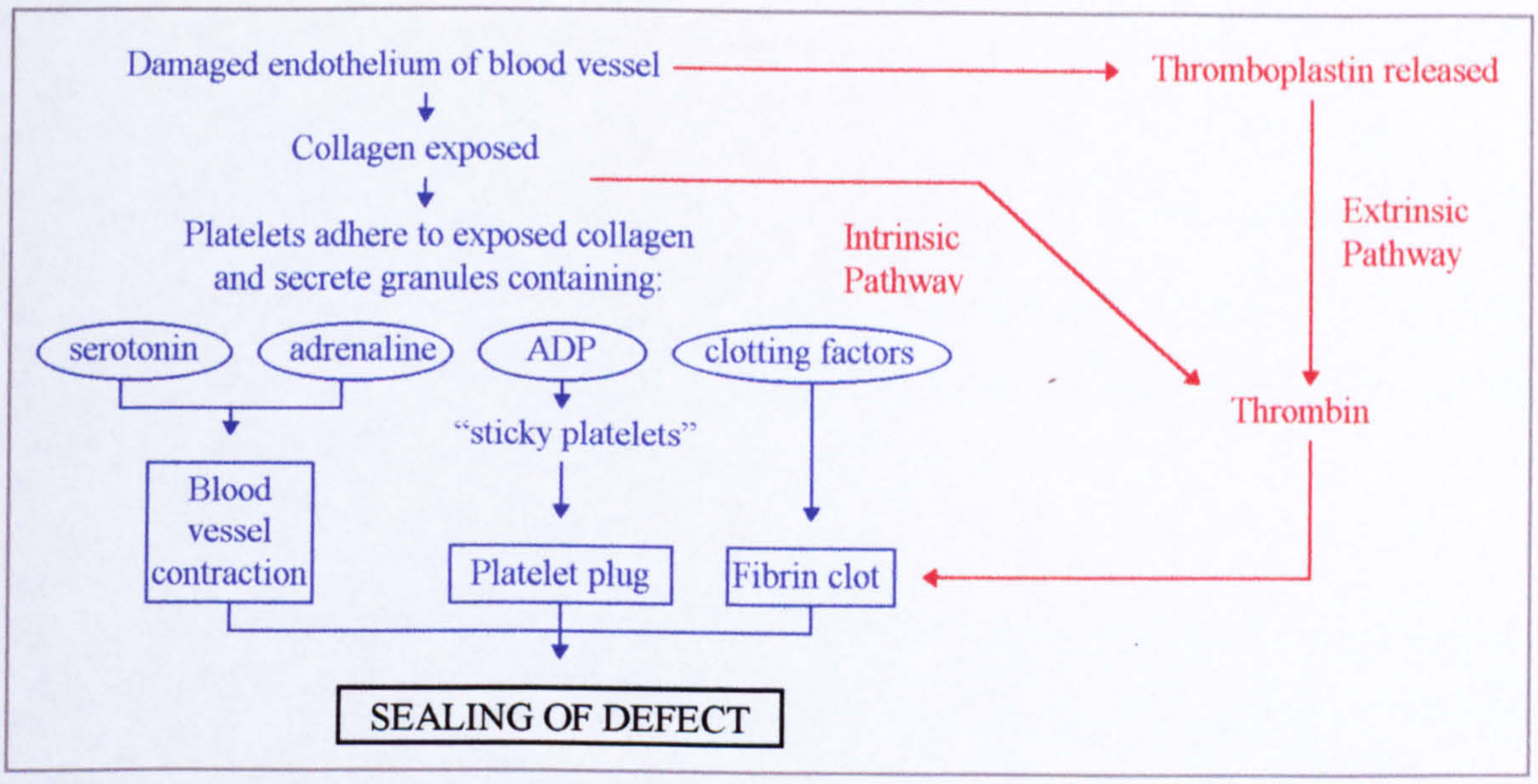


Figure 1.3: Haemostasis cascade.

(ii) Inflammatory Response

It is universally accepted that the inflammatory process plays an important role in both adhesion-free healing and adhesion formation. Inflammation is a localised protective response initiated by injury, with the aim of destroying, diluting or walling-off both the source of injury and the injured tissue. This complex process involves many biochemical, cellular and physiological processes, with the main characteristics shown in Figure 1.4. Involvement of inflammatory cells during the complete healing process of peritoneal wounds has been demonstrated by several studies, with inflammatory cells seen in healing wounds from as little as 1 hour to at least 10 days post injury.

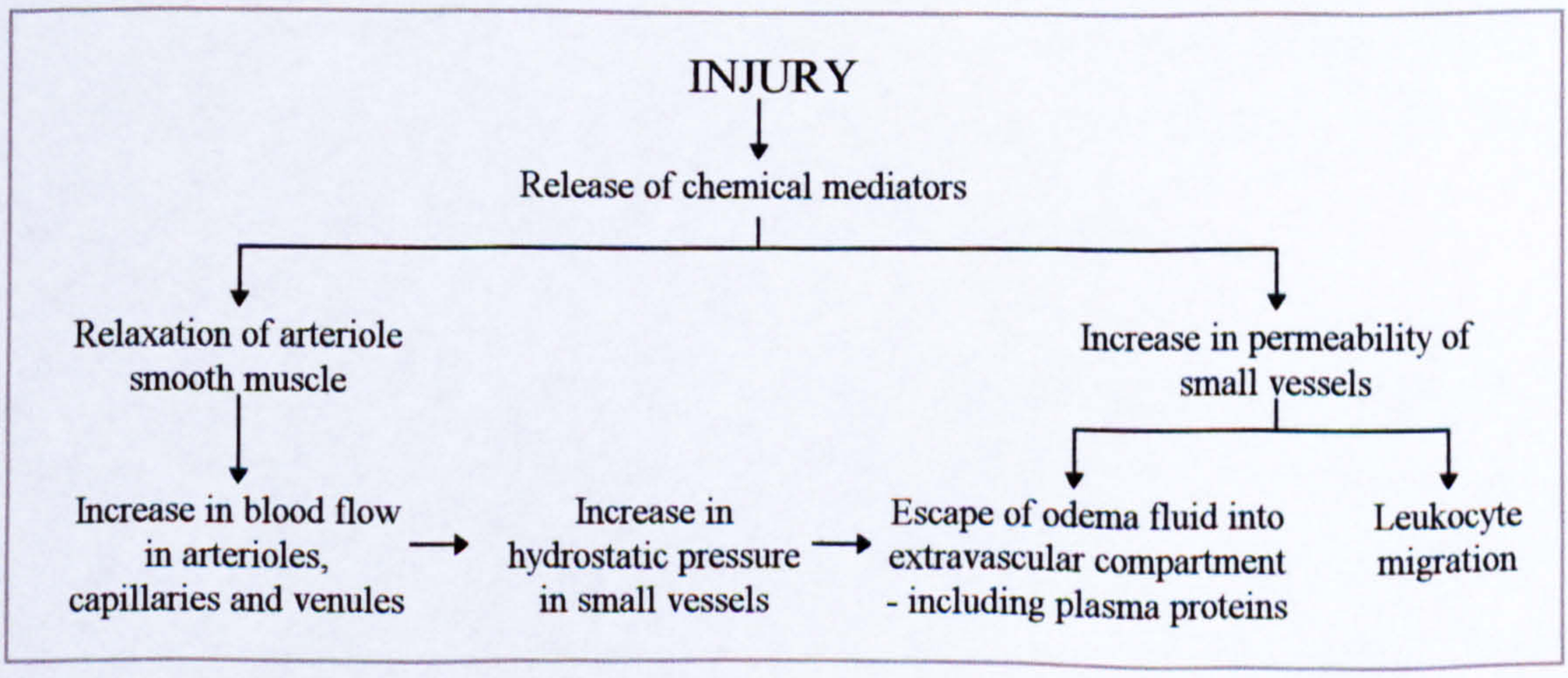


Figure 1.4 : Main characteristics of the inflammatory response.

Eskeland (1966.1; 1966.2) sampled healing tissues at 1 and 4 hours post injury, and found cells, predominantly macrophages with occasional mast cells, entangled within

fibrin strands. By 12 hours, numerous cells were present within the fibrin strands, with polymorphonuclear leukocytes (PMNLs) the predominant cell type but many macrophages, a few eosinophils and mast cells were also seen (Raftery, 1973.E). Red blood cells, together with nucleated cells (macrophages, monocytes, polymorphs, eosinophils, lymphocytes and mast cells) have also been found in the fibrinous exudate at 12 to 24 hours (Ellis *et al.,* 1965; Raftery, 1973.L).

After 24 hours underlying tissue still shows signs of damage and oedema. Neutrophils, eosinophils (Johnson & Whitting, 1962; Hubbard *et al.,* 1967; Ryan *et al.,* 1973; Raftery, 1973.E), lymphocytes, histiocytes (Hubbard *et al.,* 1967) and macrophages (Johnson & Whitting, 1962; Hubbard *et al.,* 1967; Raftery, 1973.L; Raftery, 1973.E) have all been demonstrated. However Ryan *et al.,* (1973) reported macrophages to be absent at this stage.

However studies often detail contradictory results for the involvement of inflammatory cells at day 3. This may be a function of the type of injury inflicted. Ryan *et al.,* (1973) reported that underlying tissue contained polymorphs and macrophages, whereas Raftery's studies (Raftery, 1973.L; Raftery, 1973.E) state that polymorphs, lymphocytes, eosinophils and mast cells were no longer present, with the only inflammatory cells remaining being macrophages. The same findings were presented by Hubbard *et al.,* (1967) and this view is supported by Eskeland (1966.1) who showed that a large number of closely packed macrophages were seen in the superficial parts, often contrasting sharply with the relatively small number of cells in deeper parts.

At 4 days cellular involvement is still marked, with reports of large numbers of cells present, evenly distributed throughout the wound (Eskeland, 1966.1) and considerable proliferative activity in undifferentiated mesenchymal cells, macrophages and cells forming new blood vessels in the wound floor (Johnson & Whitting, 1962). Ryan *et al.,* (1973) reported the presence of the same cell types as day 3, with the addition of underlying smooth muscle showing signs of microfilament regeneration (Ryan *et al.,* 1973). A decrease in inflammatory cells occurred thereafter and by 7 and 10 days

following injury, underlying tissue showed only scattered macrophages (Raftery, 1973.E) (Figure 1.5).

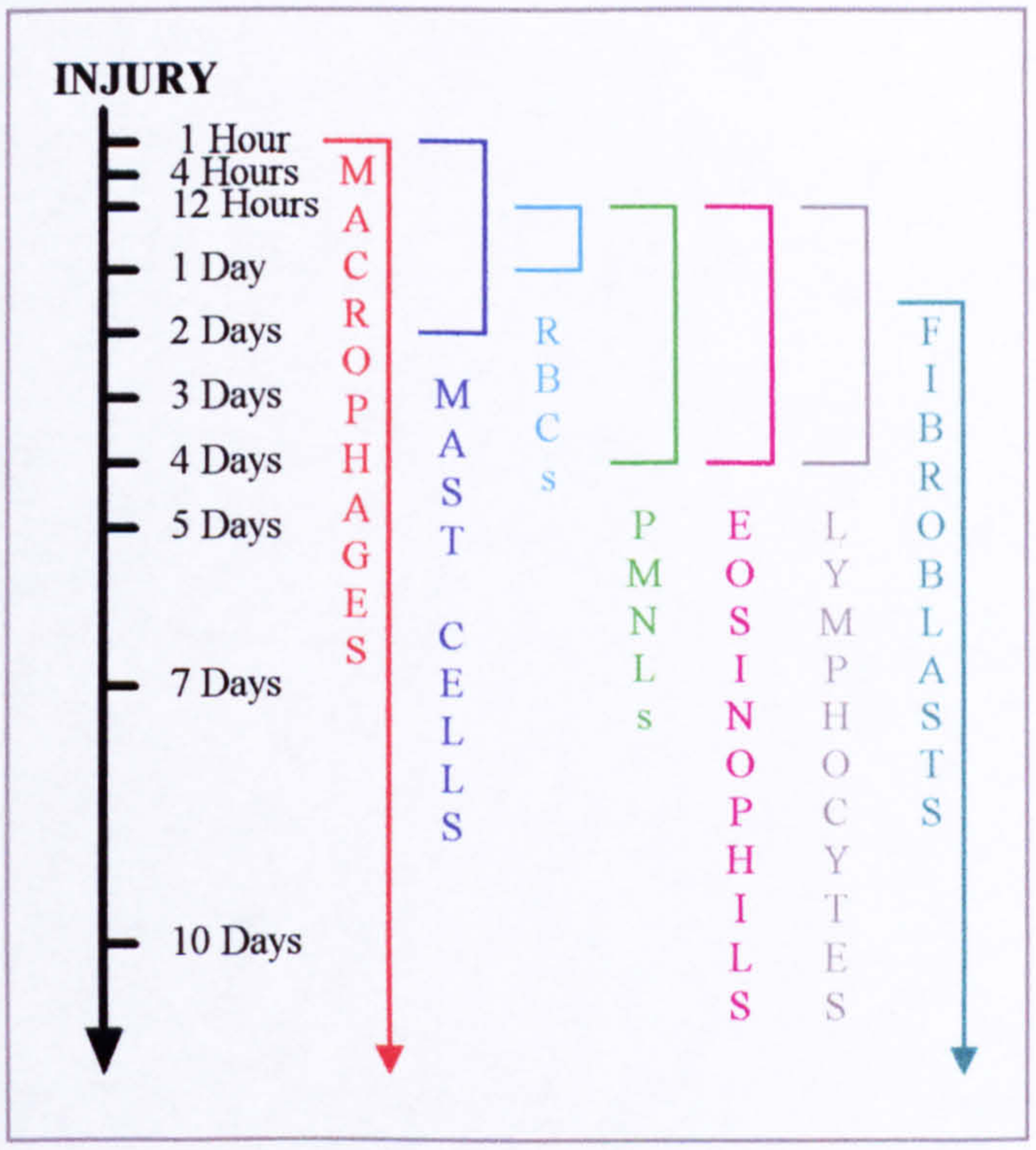


Figure 1.5: Cellular involvement during adhesion-free wound healing.

(iii) Tissue Repair

The final cellular process of wound healing is tissue repair, involving both surface and deeper areas of the wound. There is general agreement for the healing of underlying tissues. Young fibroblasts have been seen in the base of the wound as early as 2 days post injury (Ellis *et al.*, 1965) (Figure 1.5). At 4 days fibroblast proliferation has been observed (Johnson & Whitting, 1962; Raftery, 1973.L), with fibroblasts alignment parallel to the wound surface seen by 5 days (Raftery, 1973.L; Raftery, 1973, E). This increase in fibroblast number and alignment corresponds with the presence of collagen observed at 5 days following injury (Raftery, 1973.L; Ryan *et al.*, 1973). Fibroblasts have been demonstrated at 10 days following injury (Raftery, 1973.E), although Ellis *et al.*, (1965) reported that fibroblasts are replaced by histiocytes at 7 days. In addition, smooth muscle regeneration has been demonstrated as early as 4 days post injury and is reported as being completed by 7 days (Ryan *et al.*, 1973).

The question of cellular regeneration of the covering mesothelial layer on the wound surface is much more contentious (Table 1.4). There are four main theories for the origin of new mesothelial cells:

I. **Mesothelial cells**, either implanted from contact with peritoneum (Johnson & Whitting, 1962) or by proliferation and/or migration from adjacent, non-injured peritoneum (Robbin *et al.,* 1949; Eskeland, 1966.1 (small defects) Hubbard *et al.,* 1967 (minor role); Johnson & Whitting, 1962)

II. **Monocytes/macrophages** differentiation into mesothelial cells (Johnson & Whitting, 1962; Eskeland, 1966.1; Eskeland, 1966.2; Ryan *et al.,* 1973 (mononuclear cells))

III. **Fibroblast** differentiation into mesothelial cells (Ellis *et al.,* 1965; Raftery, 1973.L; Raftery, 1973.E)

IV. **Mesenchymal cell** differentiation into mesothelial cells (Hubbard *et al.,* 1967; Raftery, 1973.E; Raftery, 1973.E (via fibroblasts))

Cell Type	Author's	
	Support	**Against**
Mesothelial cell	Robbin *et al.*, 1949 Johnson & Whitting, 1962 Eskeland, 1966.1 Hubbard *et al.*, 1967	Eskeland, 1966.1 Eskeland, 1966.2 Raftery, 1973.E
Monocyte/macrophage	Johnson & Whitting, 1961 Eskeland, 1966 .1 Eskeland, 1966 .2 Ryan *et al.*, 1973	Raftery, 1973.L Raftery, 1973.E
Fibroblast	Ellis *et al.*, 1965 Raftery, 1973.L Raftery, 1973.E	
Mesenchymal cell	Hubbard *et al.*, 1967 Raftery, 1973.E	

Table 1.4: Possible origins for mesothelial cells.

The contradictory evidence for the origin of mesothelial cells is hard to interpret, but it is possible that there are several origins for regenerated mesothelium, with the pathway chosen dependent upon the injury type and peritoneal conditions. Several studies have listed different origins of new mesothelial cells in order of preference (Johnson & Whitting, 1962; Eskeland, 1966.1; Raftery, 1973.E) which support this hypothesis. Contributing factors to these contradictory theories include the diverse experimental models used, use of static time points to interpret a continuous rapidly changing process,

and the lack of clear cell identification.

Although different theories exist regarding the origin of new mesothelial cells, there is general agreement in the timing of the formation of new mesothelium on the surface of peritoneal defects. Within the first 24 hours following injury cells appear on the surface of the defect (Johnson & Whitting, 1962; Eskeland, 1966.1; Ryan *et al.,* 1973), with Ellis *et al.,* (1965) demonstrating approximately 50% of the surface covered with cells at this time. By 2 days the number of surface cells has greatly increased with most of the surface covered (Johnson & Whitting, 1962; Ellis *et al.,* 1965; Raftery, 1973.E). A continuous layer of surface cells has been demonstrated in as little as 3 days following injury (Johnson & Whitting, 1962; Ellis *et al.,* 1965), with cellular attachments seen at 4 days (Ryan *et al.,* 1973). By 7 to 8 days post injury all studies report that a continuous cell layer is present, attached to a basement membrane (Raftery, 1973.E) and by 10 to 14 days the new mesothelium is indistinguishable from the normal, adjacent peritoneum (Ellis *et al.,* 1965; Eskeland, 1966.1) .

1.1.6.4 Biochemical Modulation

Little research has investigated peritoneal healing and adhesion formation at the molecular level. Since such events are a complex sequences of cellular actions and structural changes, considerable molecular control must be present. It has been estimated that well over 100 substances are involved in the regulation of peritoneal healing (Table 1.5), with the main sources being: (i) local tissue cells, and/or (ii) peritoneal fluid (Chegini, 1997). Although wound healing involves the interplay of many cell types with capabilities of secreting various cytokines and growth factors, macrophages have been identified in many studies as the most important source of molecular substances.

Growth Factors	**Cytokines**
Transforming growth factors α and β	Interleukins
Epidermal growth factor	Tumour necrosis factor-α
Fibroblast growth factor	Granulocyte macrophage colony stimulating factor
Platelet derived growth factor	Monocyte colony stimulating factor
Insulin-like growth factor	
Heparin binding epidermal growth factor	

Table 1.5: Main growth factors and cytokines involved in wound healing.

Rong *et al.*, (1997) investigated the levels of tumour necrosis factor-β_1 (TNF-β_1), granulocyte macrophage colony stimulating factor (GM-CSF), interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) in peritoneal fluid following surgery and found levels to be maximally elevated in the first five to seven days, which corresponds with inflammatory and tissue repair responses. A similar study was carried out by Ford *et al.*, (1989) in which significantly higher levels of IL-1 and 6, TNF and monocyte colony stimulating factor (M-CSF) were detected in wound fluid and it is hypothesised that these cytokines were secreted at the site of injury to interact and promote tissue remodelling. Decrease in levels of these cytokines was demonstrated by the 13th day following wounding, which may be a result of a regulatory process of the healed wound.

It is clearly evident that wound healing is under biochemical regulation. Chegini (1997) stated that, for successful healing to occur, molecular control needs to be optimised, precise and synchronised. When inhibition, interruption or excess expression occurs, healing is either impaired (non-healing) or excessive (adhesion formation).

1.1.6.5 PSA Formation

Considering the pathogeneses of PSAs it is not surprising that much research has been directed toward this field. However in this area of medicine there has been much "armchair reasoning rather than experimental studies or accurate clinical observation" (Ellis, 1971), with a trend to prematurely leap into the development and trials of potential PSA preventative agents. Hence, even though reports of PSA have been around for over a century, many questions concerning the aetiology of PSAs still remain. It is only logical that prevention of PSAs can only be optimised when PSAs are fully understood, a view which is also held by many authors in this field (Ellis, 1971; Pijlman *et al.*, 1994).

The overall formation of PSAs is in general agreement. In the middle of this century it was demonstrated by Thrompke & Siegner (1955) that injury or inflammation lead to the outpouring of fibrin and an adhesion is formed within 3 hours. This fibrinous or

primary adhesion has two possible outcomes: (i) it undergoes fibrinolysis and is absorbed within a few days (Jackson, 1958), or (ii) it becomes organised by the ingrowth of capillaries and collagen formation and becomes an established fibrous adhesion (Figure 1.6).

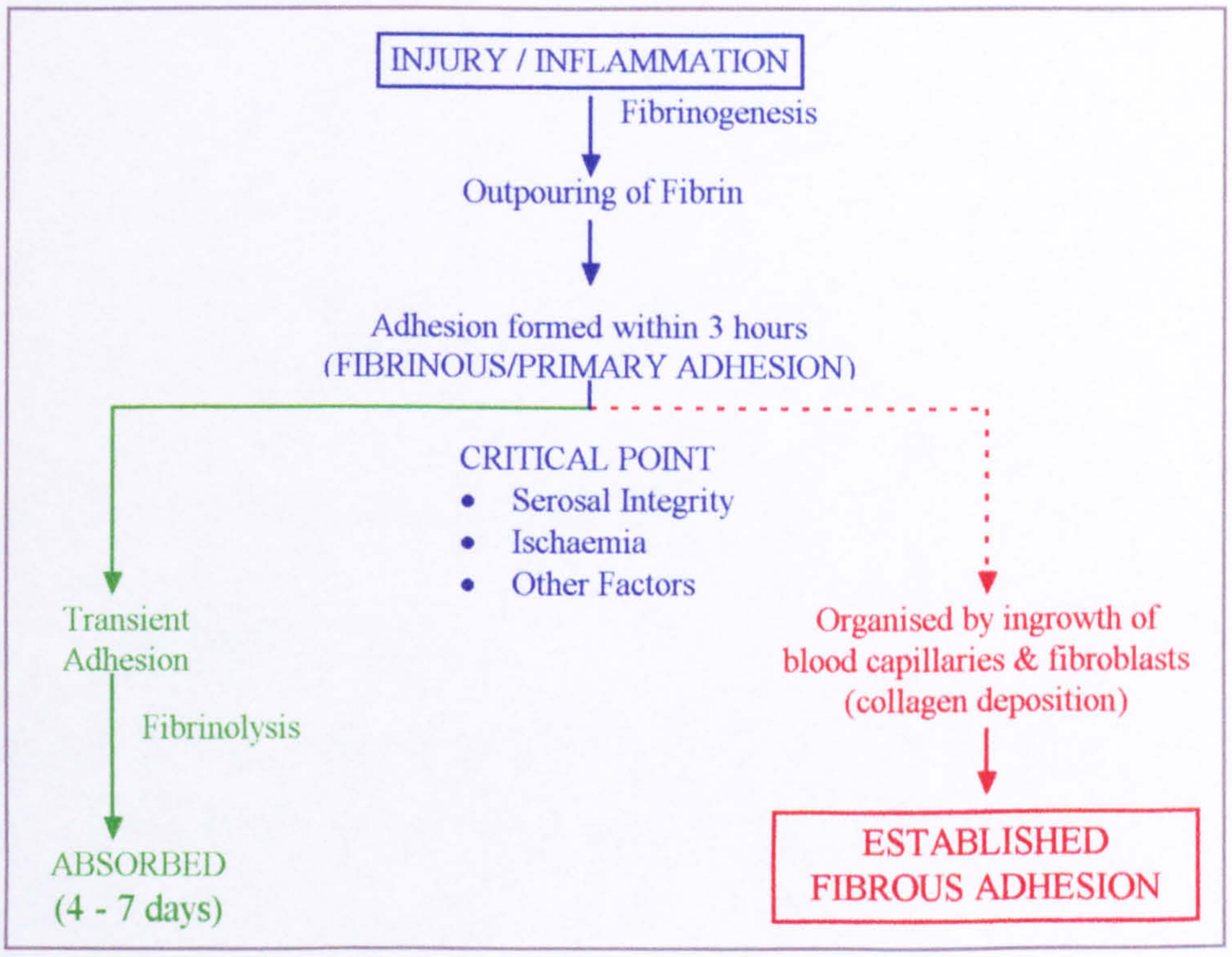


Figure 1.6: Overview of PSA formation.

1.1.6.5.1 Structural Changes

A comprehensive light and electron microscopy study carried out by Milligan & Raftery (1974) showed the dynamics of fibrin and collagen within PSAs to follow a similar pattern to adhesion-free peritoneal healing. At 1 day post injury the adhesion consisted of a fibrin matrix, which remained unchanged for the first 3 days, after which most fibrin had been removed. Sparse and fibrillar collagen was first seen at 3 days after which it became the predominant structural protein. By 5 days collagen formation was well advanced, with collagen fibres laying in discrete bundles by 7 days post injury and further maturation until the end of the 2 month study (Figure 1.7). The formation of new blood vessels was observed from 3 days onwards.

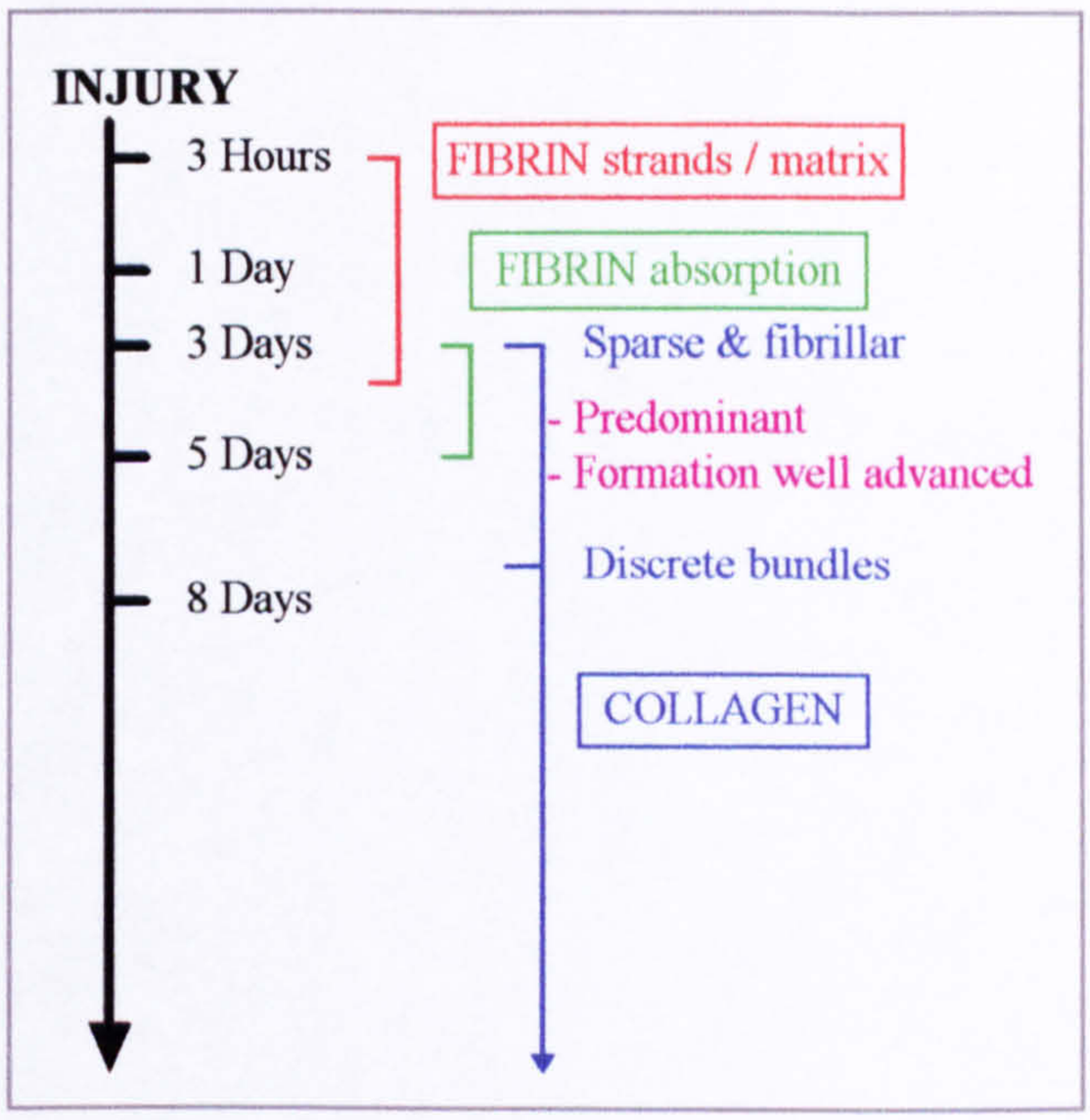


Figure 1.7: Structural changes during PSA development.

1.1.6.5.2 Cellular Involvement

As with structural development of PSAs, cellular involvement follows a similar pattern to adhesion-free peritoneal healing, that is, haemostasis, inflammatory response and tissue repair/modelling.

The study by Milligan & Raftery (1974) details cellular activity during PSA formation, with support from Schade & Williamson (1968). At 1 day cells present in the fibrin matrix were macrophages and eosinophils, distorted extravasated RBCs and necrotic cells, however PMNLs were the predominant cell type. By 2 days PMNL predominance was shared with macrophages, with lymphocytes and eosinophils also present. Reduction in the presence of PMNLs during the next 24 hours occurred leaving very few remaining by day 3, with macrophages predominant and many eosinophils present. From 4 to 7 days macrophages and eosinophils were still present, in addition to increasing numbers of fibroblasts. From 14 days onwards relatively few cells remained, consisting mainly of elongated fibroblasts interspersed with collagen bundles and occasional macrophages (Figure 1.8).

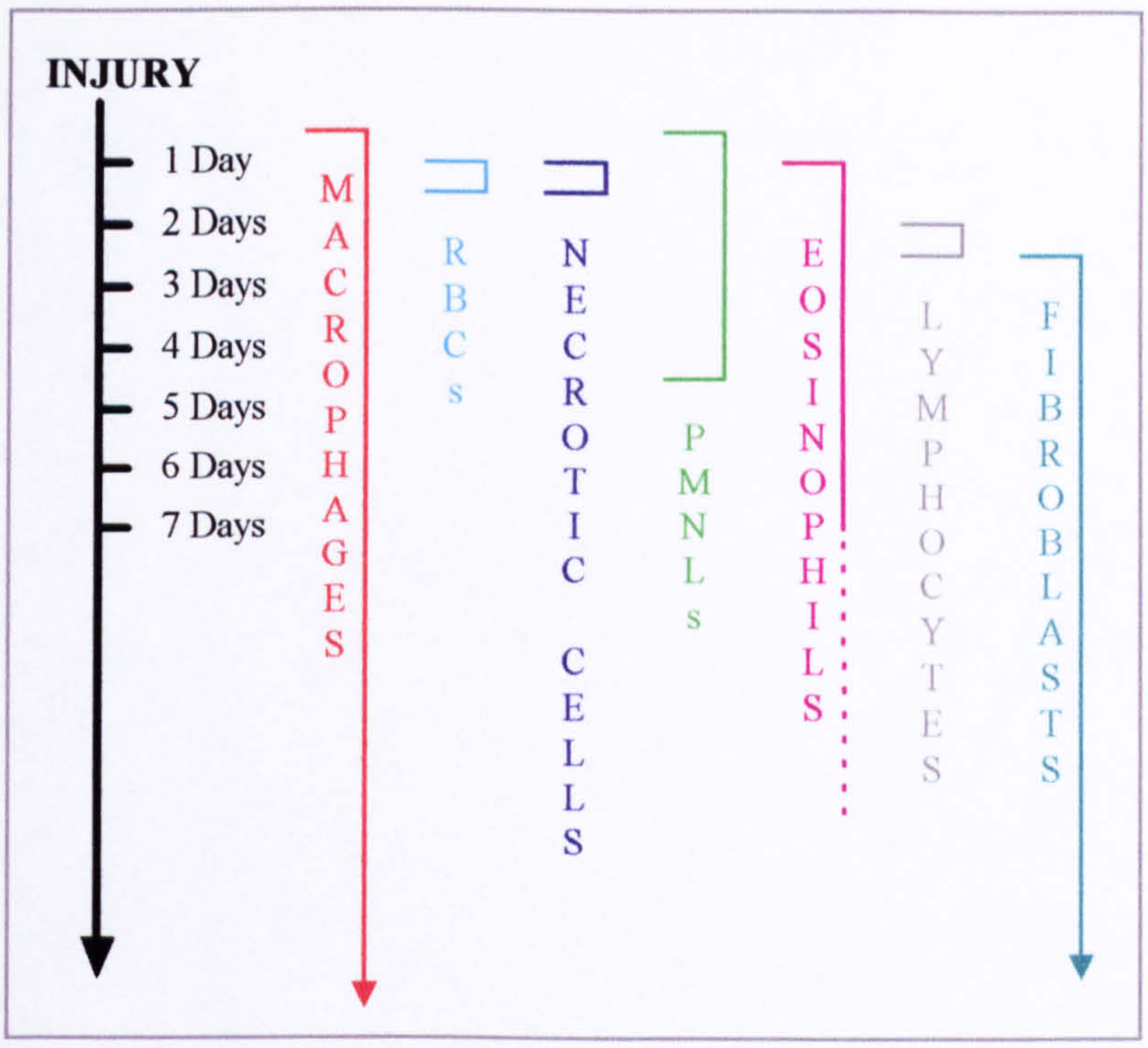


Figure 1.8: Cellular involvement in PSA formation.

1.1.6.6 PSA Formation versus Adhesion-Free Healing

The search for causative factor(s) for the formation of PSAs has been ongoing since the first description of PSAs. Many hypotheses have been put forward, some with very little scientific basis, with inappropriate ideas exacerbating the problems of research into PSAs. For example at the beginning of this century it was generally accepted that open peritoneal defects lead to PSA formation, consequently suturing of such defects was advocated. However many subsequent studies have demonstrated that peritoneal defects can heal without adhesion development (Chandy & Rhoads, 1946; Johnson & Whitting, 1962; Ellis *et al.*, 1965; Eskeland, 1966.1; Gluckson, 1966; Raftery, 1973.L; McDonald *et al.*, 1988) and that suturing of such defects acted as a potent stimulator of PSAs due to the presence of foreign material and excess handling of viscera during the repair process.

Since peritoneal defects have the potential to heal without adhesion formation it is somewhat surprising that PSAs involving peritoneal tissues are almost an inevitable outcome of surgical procedures. However, during surgery, there are many factors which have been identified as potential stimulators of PSAs (Table 1.6).

Category	Potential Stimulators of PSAs	Author's Offering Support
Mechanical Trauma		Myallarniemi & Karppinen, 1968 Schade & Williamson, 1968
	IP physiological saline solution	Chandy & Rhoads, 1946
	Exposure of viscera to air	Deaver, 1923
	Cautery	Deaver, 1923 McDonald *et al.*, 1988
	Serosal Injury and Blood	Ryan *et al.*, 1971 Cliff *et al.*, 1973
Infection		Deaver, 1923 Holtz, 1984 O'Leary & Coakley, 1992
Ischaemia		Ellis, 1962 Myallarniemi & Karppinen, 1968 Dunn & Mohler, 1990
	Venous Congestion	Belzer, 1967
Foreign Materials	Sutures	Deaver, 1923 Hubbard *et al.*, 1967 Connolly & Stephens, 1968 Holtz, 1982 McDonald *et al.*, 1988 O'Leary & Coakley, 1992
	Staples	McDonald *et al.*, 1988
	Powder from Surgical Gloves	Chandy & Rhoads, 1946 Jangelman & Ellis, 1973
	Endotoxins from Surgical Gloves	Perio *et al.*, 1990
	Dry Gauze	Connolly & Stephens, 1968
Reduction in Fibrinolytic Activity		Buckman *et al.*, 1976 Raftery, 1981 Menzies & Ellis, 1989 Vipond *et al.*, 1990

Table 1.6: Potential stimulators of PSAs.

Potential stimulators of PSAs can be divided into 5 main groups:

i. Mechanical trauma - denuding of peritoneal surfaces can occur as a result of very mild injuries (for example, drying for 5 minutes or wetting with isotonic saline solution for 30 minutes (Ryan *et al.*, 1971)) due to the intricate structure of covering

mesothelial cells. However it is generally agreed that such defects will heal successfully although perhaps not optimally, if left to heal naturally without the interference of other agents.

ii. Infection - the potential of infection to stimulate adhesion formation has long been recognised. PSA development in this situation has preventative effects in limiting infection topographically in the peritoneal cavity (Deaver, 1923). Holtz (1984) highlighted the fact that the actual mechanisms by which infection induces adhesion formation are not clearly understood but probably include the numerous substances released by bacteria, including enzymes, which may damage tissue and induce the inflammatory response and substances which limit blood flow.

iii. Ischaemia - the importance of ischaemia in PSA formation was first elucidated by Ellis (1962), with later studies supporting his hypothesis (Belzer, 1967; Myallarniemi & Karppinen, 1968; Dunn & Mohler, 1990). Ellis (1962) stated that factors which are associated with PSAs, such as tissue damage, removal and suturing, can be classified as tissue ischaemia, and that this, and not serosal damage, is the important factor in PSA development.

iv. Foreign materials - many extraneous materials commonly associated with open wound surgery have been implicated in PSA formation. The actual pathways in which such materials induce PSAs are not fully understood, but probably involve ischaemia and/or inflammatory pathways.

v. Reduction in fibrinolytic activity - many fibrinous PSAs have the potential to undergo lysis in the first week following surgery (Jackson, 1958), a phenomena well known by surgeons. Fibrin clots/fibrinous adhesions are removed by a fibrinolytic system, which degrades fibrin and fibrinogen to soluble products (Figure 1.9).

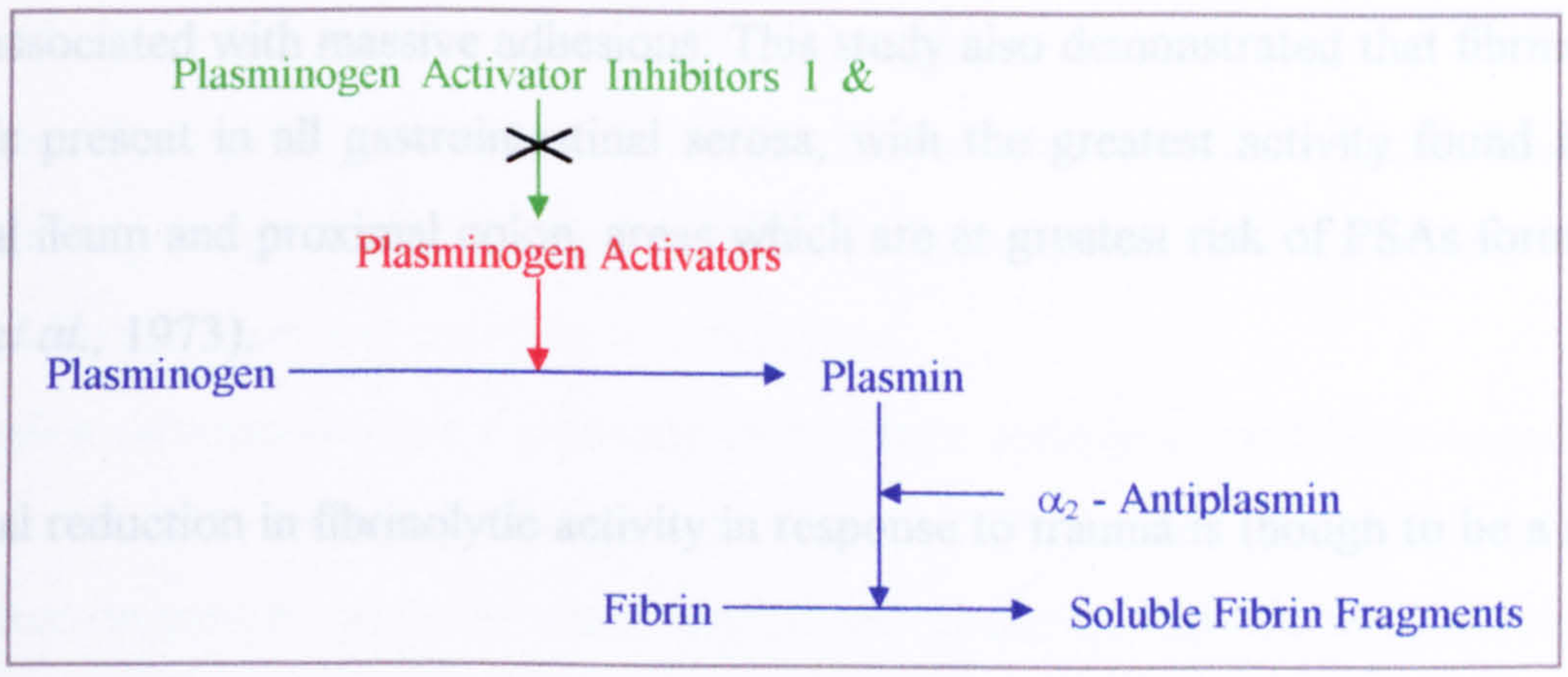


Figure 1.9: Fibrinolysis.

Many studies have demonstrated that natural fibrinolytic activity is effected by trauma, with a sustained decrease in activity up to 2 days post surgery. Ryan *et al.*, (1973) demonstrated that fibrinolysis was absent at 4 to 24 hours post surgery, with similar results found by Scott-Coombs *et al.*, (1995) in which plasminogen activating activity (PAA) was absent at 24 and 48 hours. Raftery (1981) stated that fibrinolytic activity, although reduced, was not maximally depressed at the time of operation with further depression in the first 24 hours post-operative. Following the absence of fibrinolytic ability in the first couple of post operative days, an increase in activity has been demonstrated (Ryan *et al.*, 1973), with levels remaining elevated for 2 to 3 weeks and returning to normal thereafter.

Buckman *et al.*, (1976) demonstrated that the time which it took for PAA activity to return to normal following injury was dependent upon the type of injury, with abrasion returning to normal first, followed by crushing and then ischaemia. This study hypothesised that major stimuli to intraperitoneal adhesion formation exert their effect on peritoneal fibrinolysis for several days, explaining the failure of short-term therapy to prevent PSAs. Support for this hypothesis comes from Raftery (1981) who demonstrated that significant depression of fibrinolytic activity occurred in response to peritoneal grafting, diathermy and peritoneal ischaemia.

Gervin *et al.*, (1973) showed that the relationship of adhesion production to reduction in fibrinolytic activity was highly significant, with 50% or more reduction in fibrinolytic

activity associated with massive adhesions. This study also demonstrated that fibrinolytic activity is present in all gastrointestinal serosa, with the greatest activity found in the mid-distal ileum and proximal colon, areas which are at greatest risk of PSAs formation (Gervin *et al.*, 1973).

The actual reduction in fibrinolytic activity in response to trauma is though to be a result of:

i. Reduced tissue plasminogen activator (tPA) concentration (the principal physiological PA in peritoneal tissue) (Holmdahl, 1997), and
ii. Increased concentration of plasminogen activator inhibitor 1 (PAI-1) and plasminogen activator inhibitor 2 (PAI-2) (Vipond *et al.*, 1990; Scott-Coombs *et al.*, 1995).

1.1.7 Conclusions

It is evident that the development of PSAs is dynamic and complex, involving interactions of many biological systems. However even though reports of PSAs have been around for many centuries, with their importance to both patients and medical personnel well established, many fundamental questions concerning the aetiology of PSAs still remain. It is only logical that prevention of PSAs can only be optimised when the formation and maturation of PSAs is fully understood, a view which is also held by this and many authors in this field (Ellis, 1971; Pijlman *et al.*, 1994).

1.2 Post Surgical Adhesion Preventative Strategies

1.2.1 History

Treatment of PSAs has been attempted, with varying success, since the mid-late 19th century, with early practices of adhesion division by surgery still remaining today. Naturally adhesiolysis (surgical lysis of adhesions) leads to immediate relief from PSA pathogenesis, with this practice essential for such conditions as bowel obstructions.

However there are two main problems with this technique:

i. A second surgical procedure is required, associated with additional patient discomfort, the possibility of further PSA formation (even with laparoscopic lysis) and additional economic and time expenditure

ii. Adhesion reformation rate - clinically problematic adhesions have been reported to reoccur in 32% of cases (Brightwell *et al.*, 1977), although overall adhesion reformation rate is probably much higher and closer to experimental reformation rates of 80-100% (Menzies & Ellis, 1989; Gervin *et al.*, 1973).

As with any medical condition, prevention is a much better alternative to treatment. In a comprehensive review of PSA prophylaxis, Boys (1942) identified five areas in which to develop measures against PSA formation (Figure 1.10). As recognised by Boys (1942) it is fibrinous/transient adhesions which need to be targeted, as once fibroblasts have started laying down collagen (from 3 days post operative) then only drastic measures, such as adhesiolysis and surgery, are going to be effective.

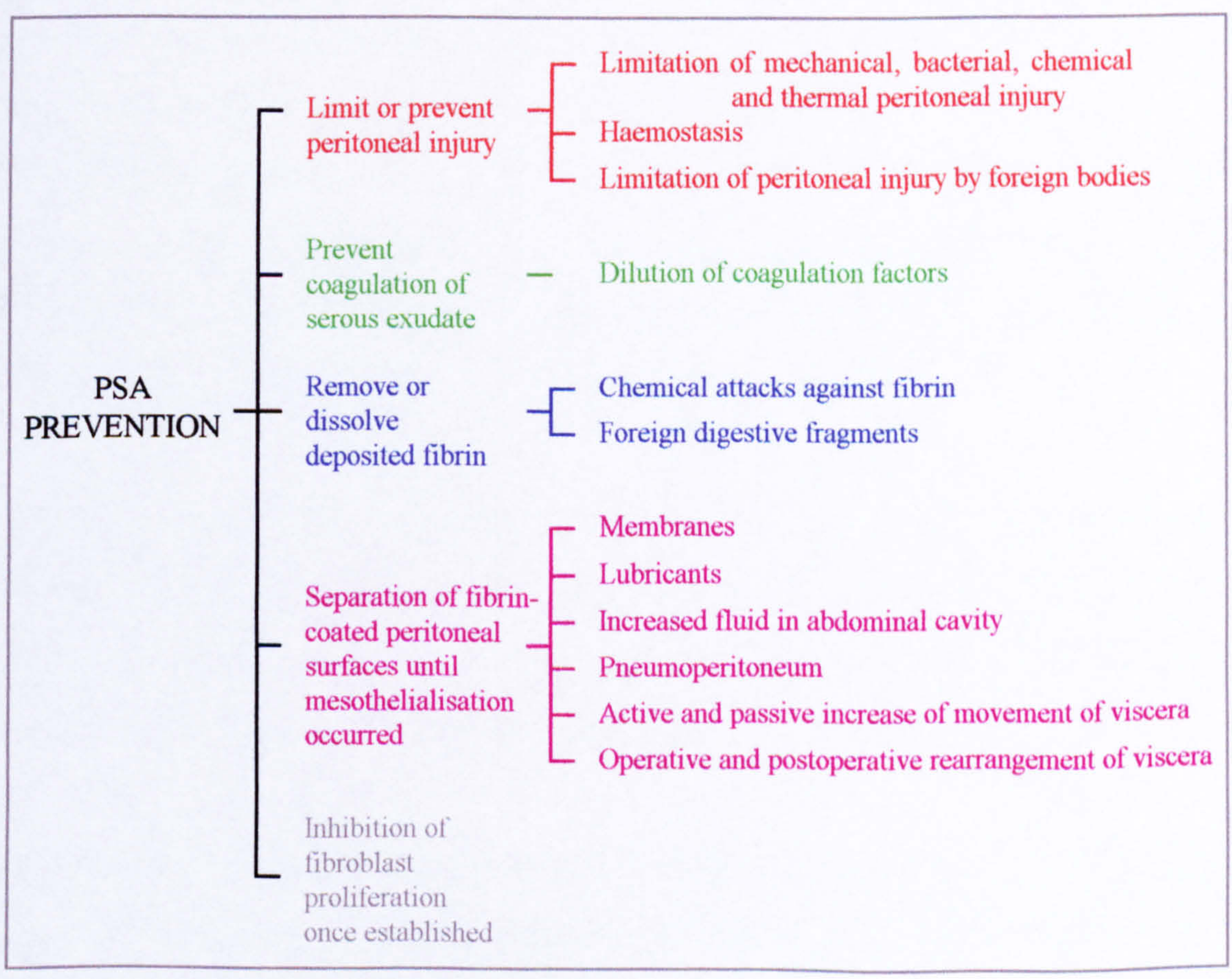


Figure 1.10: Potential PSA prevention strategies.
(From Boys, 1942).

1.2.2 Surgical Procedure Alterations

It is universally agreed that meticulous surgical technique is required to "limit" PSA formation. Such techniques includes:

1. Strict asepsis
2. Minimal, gentle tissue handling
3. Proper haemostasis/removal of "free" blood
4. Effective cautery/diathermy
5. Avoidance of foreign material contamination
6. Correct and essential only suturing
7. Influencing position of PSA formation

All of the above do not need expanding upon except the last. Many authors and surgeons agree that if PSAs are going to exist then influencing their site of formation will limit associated pathogenesis. Placement of the omentum over injured tissue and/or peritoneal suture lines are common techniques directed at prevention of intestinal adhesions and aid re-entry into peritoneum respectively. Plication techniques, such as Noble plication, Childs-Phillips plication and modifications of the Baker tube insertion are techniques devised to prevent recurrent adhesional bowel obstruction (Menzies, 1992). Again these surgical techniques are aimed at controlling the site of adhesion formation/reformation so that the bowel maintains patency and obstruction is avoided.

However it appears that suggested surgical precautions are not being optimised by surgeons, as highlighted in published surveys by Scott-Coombs *et al.*, (1993) and Holmdahl & Risberg (1997) who independently investigated current practices employed by surgeons in Britain and Sweden respectively.

It has long been recognised that starch from surgeons gloves acts as a potent stimulator of PSAs, however the use of such gloves is still widespread (Scott-Coombs *et al.*, 1993; Holmdahl & Risberg, 1997), with starch contamination during abdominal surgery common (Jangelman & Ellis, 1973; Holmdahl & Risberg, 1997). Only small amounts

of powder are required in the presence of peritoneal defect to form adhesions (Jangelman & Ellis, 1973) and hence the use of these gloves is potentially very hazardous. Although approximately half of the surgeons who use these gloves wash them prior to use (Scott-Coombs *et al.*, 1993), washing techniques employed tended to be inadequate, with simple washing in water leaving about 10% of the original starch (Fraser, 1982) and causing clumping of particles which in turn hamper absorption and lead to granuloma and adhesion formation (Jangelman & Ellis, 1973; Scott-Coombs *et al.*, 1993; Holmdahl & Risberg, 1997). Correct washing of gloves in povidone-iodine surgical scrub for one minute, followed by washing in running sterile water to remove 99.8 - 100% of starch (Fraser, 1982) was found to be utilised by only 2.6% of surgeons (Scott-Coombs *et al.*, 1993). However, even with effective washing procedures the risk of contamination via glove perforation still remains.

Both studies also reported that the majority of surgeons suture the peritoneal layer, even through this process is unnecessary (Connolly & Stephens, 1968; Ellis & Heedle, 1977; O'Leary & Coakley, 1992), time consuming and induces PSAs.

Full employment of "PSA limiting surgical practices" do not appear to be common place, with the development of avoidable PSAs occurring. Surgeons need to exploit these areas in order to lower PSA associated problems. For example, Holmdahl & Risberg (1997) estimated that the use of powder-free gloves alone could give a potential saving of US$3 million/year. However even if all "PSA limiting techniques" were utilised, it is well recognised that PSAs will still develop. Hence for many years surgeons have sought additional prophylaxis to combat this pathogenesis.

1.2.3 Potential PSA Preventative Agents

There have been many studies investigating potential PSA preventative agents, ranging from foils of silver to fish bladders. Progression in finding an effective PSA preventative agent has been slow, with inadequate understanding of PSA development hindering the process.

An "ideal" PSA preventative agent would possess the following properties:

- non toxic
- non reactive/non immunogenic
- no/limited side effects
- effective
- remain *in situ* whilst required but ultimately biodegradable
- economically viable
- easy to use/time viable

In order to assess previously investigated potential PSA preventative agents (Appendix I Table 1.3), they have been grouped into categories according to their main mode of action, following the plan laid down by Boys (1942). However these are not definitive, with some potential agents having several modes of action and not all actions known.

1.2.3.1 Prevention of Fibrin Coagulation

Substances investigated with the potential to prevent the initial stage of PSA formation, fibrin deposition, include **anticoagulants**, such as sodium citrate, heparin, Dicumorol®, and **dextran**. (Ellis, 1971; Ellis, 1982).

Of these **heparin** has been the most extensively studied, with systemic or intraperitoneal administration being effective in preventing PSAs. However treatment with heparin carries serious risk of haemorrhage (Pijlman *et al.*, 1994), with associated deaths (Connolly & Smith, 1960; Ellis, 1971). For this reason the clinical use of heparin for PSA prevention was abandoned in the late 1940's (Connolly & Smith, 1960).

1.2.3.2 Reduction of Inflammatory Response

Corticosteroids inhibit:

i. early inflammatory response - fibrin deposition (Pijlman *et al.*, 1994), decrease vascular permeability alterations, stabilise lysosome membranes and inhibit synthesis and release of histamine and other mediating substances (Holtz, 1984)

ii. later stages of wound healing - proliferation of capillaries and fibroblasts, deposition of collagen (Pijlman *et al.*, 1994) although stimulates human fibroblast growth (Holtz, 1984).

Most studies demonstrate that corticosteroid efficacy in reducing PSAs is uncertain (Connolly & Smith, 1960; Menzies, 1992; DiZerega, 1994; Pijlman *et al.*, 1994; Risberg, 1997). Clinical use has also been limited by the associated side effects, namely delayed wound healing, immunosuppression, bone and muscle pain and psychiatric disturbances after completion of treatment (DiZerega, 1994; Pijlman *et al.*, 1994; Risberg, 1997).

Non-steroidal anti-inflammatory drugs (NSAIDs) change the metabolism of arachidonic acid by altering the cycloxygenase enzyme activities and formation of their end products, including prostaglandins and thromboxane, with arachidonic acid metabolites mediating many aspects of the inflammatory response (DiZerega, 1994). Several NSAIDs have been tried including ibuprofen, indomethacin, oxyphenbutazone, with conflicting results found with regard to PSA prevention (DiZerega, 1994; Pijlman *et al.*, 1994)

Histamine antagonists, for example, promethazine, have been used in conjunction with corticosteroids (Holtz, 1984; Pijlman *et al.*, 1994) with results indicating that although this combination reduces the incidence of adhesions, it is associated with significant morbidity and mortality (Grosfel *et al.*, 1973). Promethazine is thought to uniquely limit the effects of histamine, inhibit the increased vascular permeability induced by histamine and other factors, stabilise lysosomes and may inhibit fibroplasia (Holtz, 1984). No study has evaluated the use of this drug alone (Holtz, 1984; Pijlman *et al.*, 1994).

Other substances tried include calcium channel blockers, progesterone and colchicine. **Calcium channel blockers**, for example, nifedipine hydrochloride, have been shown to reduce the degree and incidence of PSAs (Menzies, 1992; Pijlman *et al.*, 1994) probably due to an anti-inflammatory effect (Pijlman *et al.*, 1994). **Progesterone** has also been reported to have anti-inflammatory and immunosuppressive properties, although PSA prevention studies have yielded inconclusive results (Pijlman *et al.*, 1994). **Colchicine** is

a known anti-inflammatory, largely utilised in the treatment of gouty arthritis. It inhibits the release of histamine-containing granules from mast cells, has anti-mitotic properties and has been demonstrated to reduce adhesion formation (Pijlman *et al.*, 1994).

1.2.3.3 Removal/Dissolution of Deposited Fibrin

Many **fibrinolytic agents** have been investigated with regard to PSA prevention, for example, tissue-type plasminogen activator (tPA), streptokinase, streptodornase, single-chain urokinase, actase and elastase with varying results (Ellis, 1971; Pijlman *et al.*, 1994; Risberg, 1997). All these agents carry the risk of impaired wound healing (Risberg, 1997). The use of tPA has received a lot of interest with many studies reporting PSA reduction (Menzies & Ellis, 1989; Dunn and Mohler, 1993). tPA has several advantages over most other fibrinolytic agents due to it not being antigenic and has few, if any, general side effects (Pijlman *et al.*, 1994).

Other substances investigated include digestive and proteolytic enzymes. **Digestive enzymes**, for example, pepsin and trypsin, are rapidly neutralised by peritoneal exudates and hence their potential for preventing PSAs is very limited (Ellis, 1971). The proteolytic enzyme **papain** and later its derivatives (for example, Papase) attracted attention for possible PSA prevention in the first half of this century, however Boys (1942) demonstrated that this too is neutralised by peritoneal exudate and hence efficacy is unlikely. **Hyaluronidase** digests the connective tissue component hyaluronic acid and has shown some PSA reduction properties.

Removal of fibrin deposits by peritoneal lavage with saline, dextran or hypertonic dextrose solutions has also been tried. However, these are rapidly absorbed by the peritoneum and are unlikely to prevent PSAs (Ellis, 1971).

1.2.3.4 Separation of Surfaces

1.2.3.4.1 Physical Methods

Two physical methods of separation are :

1. distension of the peritoneal cavity (Boys, 1942; Ellis, 1982), and,
2. early and increased peristalsis (Boys, 1942; Connolly & Smith, 1960; Ellis, 1982)

Although these help prevent contact of peritoneal surfaces they are generally impractical, and consequently have stimulated little clinical interest.

1.2.3.4.2 Solutions

Two types of solutions have been used in an effort to prevent PSAs:

1. large quantities of **isotonic solutions**, or,
2. smaller quantities of **hypertonic solutions** (which has the potential to form enough transudate)

Solutions investigated include normal saline, lactate Ringer's solution, hydroxyethyl starch, gelatine, silicone, povidone-iodine/polyvinylpyrrolidine, phosphate buffered saline (PBS) and dextran (Ellis, 1971; Menzies, 1992; DiZerega, 1994), usually administered as an instillate at the end of the surgical procedure (DiZerega, 1994). Although hydroflotation is effective in the early stages of wound healing, solutions tend to be absorbed before the completion of peritoneal healing, hence their preventative effects against PSA formation is dubious.

The most extensively studied solution is **dextran**, a water soluble polysaccharide, as a hypertonic solution, originally employed as a plasma expander. Low molecular weight dextran yielded insignificant results for PSA prevention and an unenthusiastic response from clinicians. However high molecular weight (HMW) dextran (mainly 32% dextran 70), although it too has produced mixed results in experimental and clinical studies, has attracted more interest. Nowadays HMW dextran is the most commonly used substance employed to separate mesothelial surfaces (Pijlman *et al.*, 1994), although it has not gained regulatory approval for adhesion prevention (Risberg, 1997). It is thought to have

multiple mechanisms of action (Pijlman *et al.*, 1994; Risberg, 1997):

- hydroflotation and siliconisation to separate peritoneal surfaces
- dilution to diminish local fibrin concentration
- preservation of local plasminogen activators
- interference with polymorphonuclear neutrophil expression of adhesion molecules
- fibrin alterations, making it more susceptible to degradation

Dextran is associated with some common side effects, including anaphylactic shock, allergic reactions, transient weight gain, ascites, oedema of the vulva or leg, pleural effusion, coagulopathy, fluid imbalance secondary to intraperitoneal osmotic load, impaired haemostasis, infection and transient liver disturbances reported (Holtz, 1984; DiZerega, 1994; Pijlman *et al.*, 1994; Risberg, 1997).

1.2.3.4.3 Physical Barriers

Placement of a physical barrier between injured peritoneal surfaces prevents contact of such surfaces and thus has the potential to prevent PSA formation. Types of barriers investigated can be classified as either natural or artificial, with further division into non-absorbable and absorbable.

Natural Barriers

The introduction of natural, theoretically absorbable membranes was seen in the 1930's (Boys, 1942). Since then many natural barriers have been investigated with the aim of preventing PSAs, including amniotic membrane, omentum, ox peritoneum, beef allantoic membrane, fish bladder and mesothelial grafts (Boys, 1942; Connolly & Smith, 1960; Ellis, 1982; Pijlman *et al.*, 1994). However such barriers usually promote PSA formation rather than prevent it (Pijlman *et al.*, 1994).

Artificial, Non-Absorbable Barriers

Artificial, non-absorbable barriers were introduced in the first half of this century with many materials investigated, including foils of silver and other metals and sheets of silk

and rubber (Boys, 1942; Ellis, 1982). Unsurprisingly instead of preventing adhesions, these materials promoted adhesion formation, probably primarily by the foreign material pathway.

Nowadays there is only one main non-absorbable material used for potential PSA prevention, Gore-Tex Surgical Membrane (W.L. Gore & Associates, Flagstaff, AZ, USA). This is an expanded polytetrafluoroethylene 0.1mm thick sheet, used as a substitute for the pericardium (DiZerega, 1994). It has small pores (≤1μm) which retard cellular penetration and are thought to contribute to the lack of tissue adherence (DiZerega, 1994). Properties important to its potential PSA preventing ability include (DiZerega, 1994; Risberg, 1997) that it is non-biodegradable, non-thrombogenic, non-reactive/ non-immunogenic, with poor tissue adherence and easy handling at laparotomy. However, due to its poor tissue adherence Gore-Tex surgical membrane needs to be anchored in place, usually via sutures, hence remains as a permanent foreign material (DiZerega, 1994; Risberg, 1997). With regard to its PSA prevention power, conflicting reports have been made, however most clinical studies have recorded PSA prevention (DiZerega, 1994; Pijlman *et al.*, 1994).

Artificial, Absorbable Barriers

Oxidised Regenerated Cellulose (ORC) is a rayon patch which has been treated with nitrogen tetraoxide. Characteristics include that it is non-reactive, persists during the critical stages of re-epithelialisation then undergoes absorption and has antibacterial properties.

The two main ORC barriers investigated for PSA prevention are:

1. **Surgicel®** (Johnson & Johnson Medical Inc., Arlington, TX, USA) an absorbable material used as haemostat. Once *in vivo* it is converted into gelatinous mass and absorbed within a few days. Studies have shown that Surgicel® both increase and prevent PSAs (DiZerega, 1994; Pijlman *et al.*, 1994).

2. **Interceed®** (Johnson & Johnson Medical Inc., Arlington, TX, USA) is similar to Surgicel®, although has a longer residence time (DiZerega, 1994). It undergoes

hydrolysis into a gelatinous "cocoon" within 8 hours (DeCherney & DiZerega, 1997), breaking down macroscopically within 3 to 10 days and microscopically within 28 days (DeCherney & DiZerega, 1997; Risberg, 1997). In order to optimise the use of Interceed®, intraperitoneal irrigants and excess peritoneal fluid need to be removed and adequate haemostasis achieved (DeCherney & DiZerega, 1997) to ensure it maintains its position within the peritoneum without the use of sutures. The efficacy of Interceed® has been shown to vary (DiZerega, 1994; Pijlman *et al.*, 1994; DeCherney & DiZerega, 1997) however its acceptance has been constrained by its decreased efficacy in the presence of blood or excess peritoneal fluid (Risberg, 1997) and although it is used in a wide variety of general surgical procedures in some countries, but not in the United States as it is not FDA approved.

Seprafilm™ (Genzyme Corporation, Cambridge, MA, USA) is a hyaluronic acid-carboxymethylcellulose (HA-CMC) polymer, which is non-toxic, non-immunogenic, biodegradable and biocompatible (Risberg, 1997). Once *in vivo*, Seprafilm™ turns into a hydrophilic gel within one day and forms a protective tissue coat for up to 7 days. The HA component is cleared from the body in 28 days, although the rate of CMC clearance is less clear (DeCherney & DiZerega, 1997; Risberg, 1997). Studies have shown that Seprafilm™ reduces PSA formation (DeCherney & DiZerega, 1997) and has gained regulatory approval in many European countries and North America (FDA approval) for PSA reduction. Seprafilm™ does not require suturing to remain in place and is effective in the presence of blood (Risberg, 1997), however care needs to taken with its application. It must be kept dry (excess fluid aspirated), handled gently with dry instruments and/or gloves and contact with tissues surfaces avoided until the application site reached. If incorrect contact occurs, standard irrigation solution is used to dislodge the membrane (DeCherney & DiZerega, 1997).

Other barriers assessed include hyaluronic acid (HA) which forms a viscous solution which appears to coat peritoneal surfaces and has had some success in reducing PSAs (Pijlman *et al.*, 1994).

Fibrin sealants: The use of fibrinogen as a haemostat has attracted attention for over a century, although it was not until the 1940's that its use as a tissue adhesive was introduced (Sierra, 1993). The early concept of fibrin glue, however, failed to flourish due to lack of strength and low efficacy of the glue, which in turn was due to poor technology resulting in low fibrinogen concentrations (Adamyan *et al.*, 1991; Sierra, 1993). In the 1970's cryoprecipitation techniques were used to obtain elevated levels of fibrinogen and other plasma proteins, which were used in conjunction with bovine thrombin with some success for nerve anastomoses (Sierra, 1993). Since then the interest in **fibrin glues or sealants** has thrived and nowadays surgical applications for fibrin sealants primarily include haemostasis and tissue sealing but also anastomoses and wound healing (promoting normal wound healing and PSA prevention).

Fibrin sealants are tissue adhesives derived from blood, which exploit the final stages of coagulation. They generally consist of two component solutions:

- component 1: fibrinogen (human), together with factor XIII
- component 2: thrombin (human or bovine), together with calcium chloride

Mixing of these during application causes the formation of the fibrin sealant (Figure 1.11). The sealant adheres to many surfaces, for example, exposed collagen at wound sites, sealing them from the surrounding environment. The sealant will remain *in vivo* for several days, aided by the addition of an anti-fibrinolytic agent (for example, aprotinin), after which it will be absorbed by enzymatic and phagocytic mechanisms.

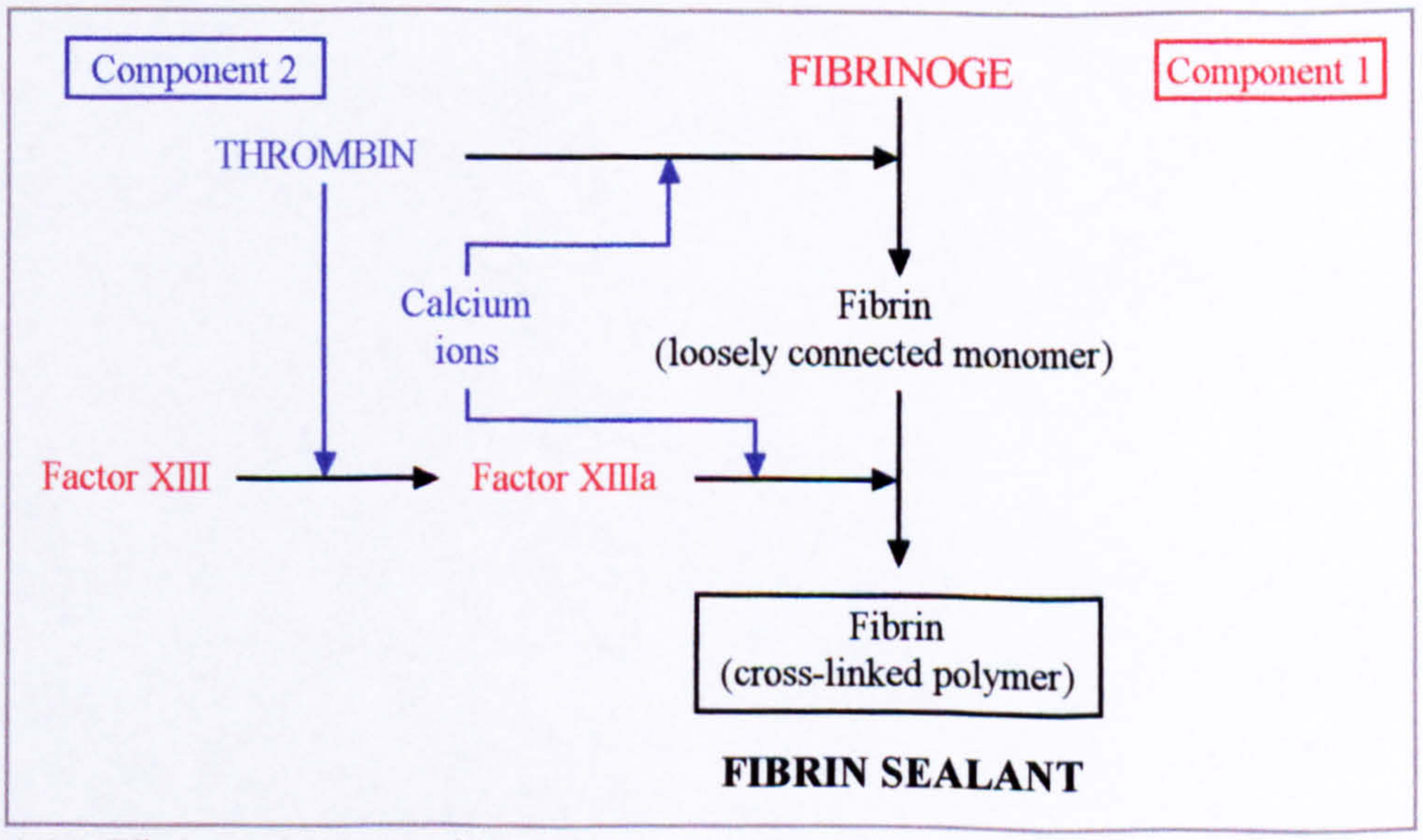


Figure 1.11: Fibrin sealant genesis.
(Adapted from Sierra, 1993).

The most commonly used application system for fibrin sealants is the Duploject double syringe system. (Figure 1.12). The contents of the two syringes are released at the same time and mixed in the Y-piece of the syringe, where polymerisation is initiated, and in this way fibrin sealant can be directly applied to the site.

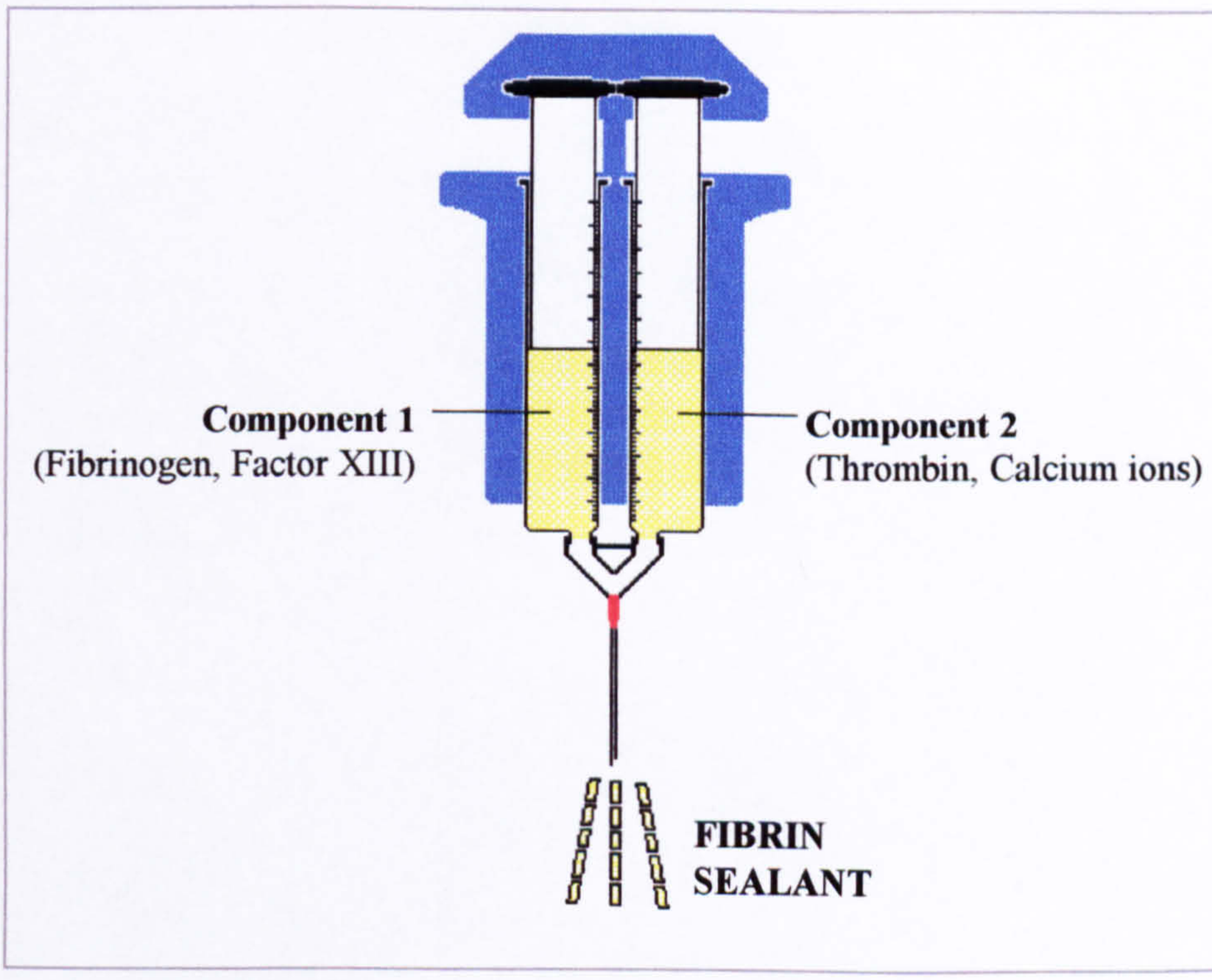


Figure 1.12: Duploject fibrin sealant application system.

Commercially available fibrin sealants include Tisseel® or Tissucol® (Immuno AG, Vienna, Austria), Beriplast® (Behringwerke, Marburg, Germany) and Biocoll® (Centre de Transfusion Sanguine de Lille, France) which utilise human pooled fibrinogen. Although these, like all blood products, are carefully screened for viral contaminates, regulatory approval for clinical trials have only been gained in Europe, with the exception of Tissucol® which has also been approved in Japan and recently the USA.

1.2.3.5 Inhibition of Fibroblast Proliferation

Substances which have been investigated to potentially prevent PSA formation which have fibroblast inhibitory effects include antihistamines (example: Histadyl®), steroids, cytotoxic drugs (Ellis, 1971; Ellis, 1982).

1.2.4 Conclusions

It is universally agreed that meticulous surgical technique can limit the formation of PSAs, reducing the risk of adhesion associated pathogeneses. Full implementation of these techniques needs to occur in general surgery to preclude the formation of preventable PSAs.

Much time and resources have been directed to find an ideal PSA preventative agent due to the medical and economic problems associated with PSAs. Despite such efforts there is currently no commercially available PSA preventative agent. As pointed out by Ellis (1982) there is a typical pattern associated with these agents: initial enthusiasm leading to clinical cases/trials, followed by further investigations showing either no effect or increased PSA formation compared to controls, finally resulting in substance being abandoned.

There are several contributing factors responsible for this:

- lack of understanding of the physiological, biochemical and cellular mechanisms governing PSA formation
- premature trials resulting from observations rather than hard scientific research
- lack of uniformity in PSA assessment model - variation in animal species, adhesion stimulus, severity, site and treatment itself (drug dose, administration route)
- qualitative assessment of PSAs and PSA prevention leading to potentially biased, non-standardised and non comparable results with other studies

Until these problems are dealt with it is unlikely that a PSA preventative agent will be found.

1.3 Vivostat™ System Human Derived Fibrin Sealant

1.3.1 Commercially Available Fibrin Sealants

Although fibrin sealants represent one of the most promising surgical develops of the last

decade, with uses in haemostasis, tissue sealing and PSA prevention, there are still problems associated with this therapy. Commercially available fibrin sealants are derived from pooled human plasma together with exogenous agents such as thrombin, Factor XIII, calcium ions and in some sealants, anti-fibrinolytic agents such as aprotinin. As discussed in section 1.2.3.4.3, due to the use of pooled human fibrinogen many such products have failed to obtain FDA approval for use in the United States. However the risk of viral contamination from human fibrinogen is not the only clinical risk associated with these products. The use of bovine products, such as bovine thrombin and aprotinin have risks of bovine spongiform encephalopathy (BSE) and antigenic reactivity (Hughes & Westwood, 1994).

In order to totally eliminate the potential risks of infection and antigenicity, two parameters need to be met:

1. eradication of all donor derived viruses or infectious agents
2. no exogenous proteins added

Due to the difficulty of blood product licensing and FDA approval in the United States, the use of autologous fibrinogen in fibrin sealants has been investigated. Fibrinogen can be successfully obtained from blood by the process of cryoprecipitation. However this procedure has proved to be technically non-viable, due to the long preparation time and low yield. The elimination of exogenous proteins however has received less attention, due to their clinical acceptability, even though potential problems remain.

1.3.2 Autologous, Human Derived Fibrin Sealant

There are many references in the literature reporting the use of autologous fibrin sealants, however this definition is inaccurate. These sealants utilise autologous human fibrinogen, together with non-autologous proteins (thrombin, aprotinin), hence these sealants are better referred to as semi-autologous fibrin sealants (Cederholm-Williams, 1994). At present there is no truly autologous fibrin sealant available commercially.

In view of problems associated with currently available fibrin sealants, ConvaTec Ltd

(A Bristol-Myers Squibb Company) have successfully developed a novel, truly autologous, human derived fibrin sealant essentially free from foreign proteins. It uses the serine proteinase batroxobin, bound to biotin, to cleave autologous fibrinogen to form an acid soluble fibrin I polymer, which readily dissolves in acid and repolymerises on neutralisation. On increasing the pH in the presence of calcium ions the fibrin I solution polymerises and endogenous prothrombin is cleaved to thrombin. Fibrinopeptide B is cleaved from fibrin I to form fibrin II. Thrombin also activates factor XIII, resulting in the formation of chemically stable fibrin II polymer (Edwardson *et al.*, 1994). Avidin, immobilised to an inert support agarose, is added to the fibrin I solution, binding to the biotin-batroxobin and the resulting complex is removed via filtration (Bayer *et al.*, 1994) (Figure 1.13).

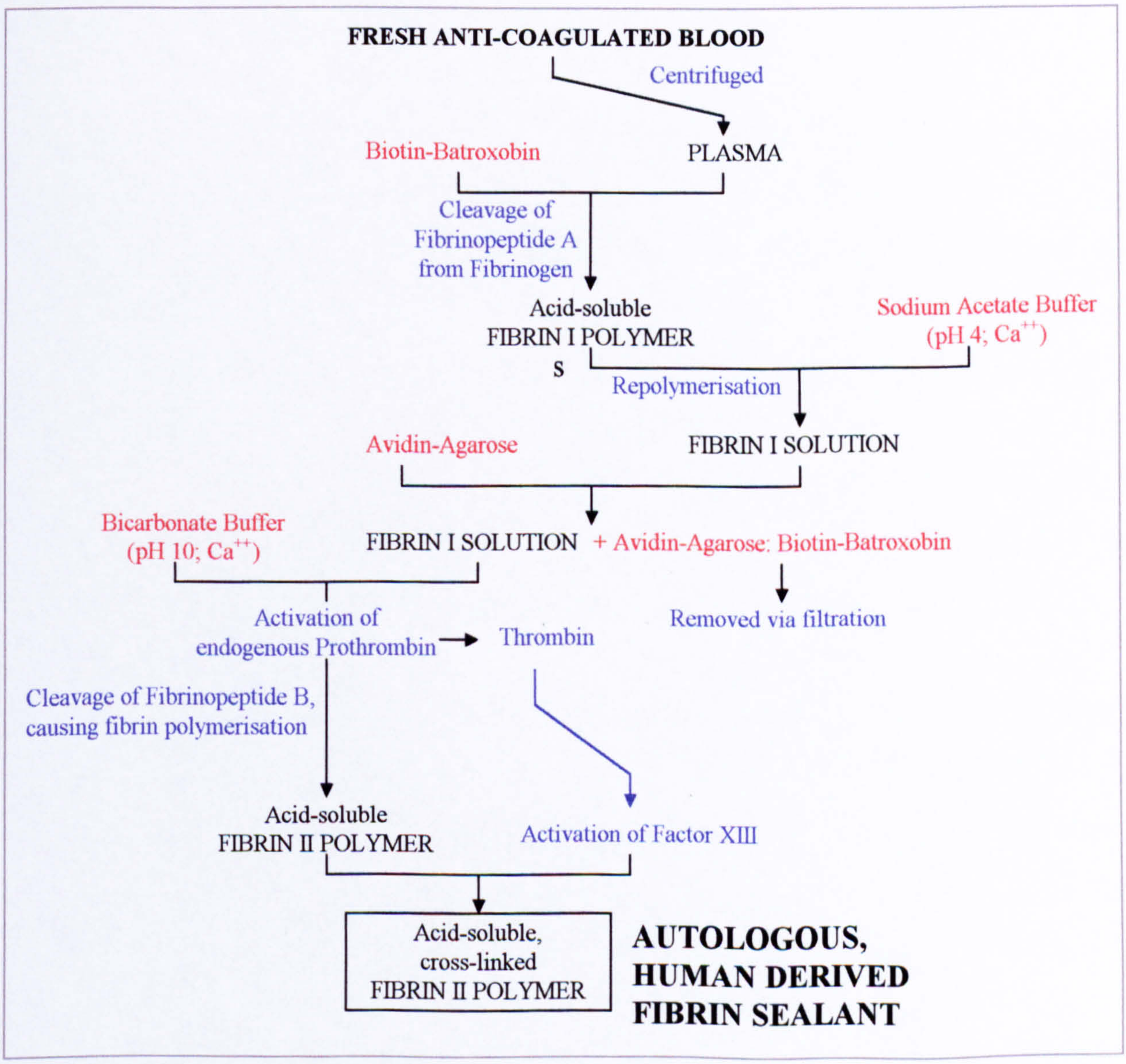


Figure 1.13: Vivostat™ system human derived fibrin sealant (novel fibrin sealant) formation (adapted from Edwardson *et al.*, 1994).

From 120ml of whole blood, 4 to 6ml of highly concentrated (20±2mg/ml) Vivostat™ system human derived fibrin sealant (novel fibrin sealant) is prepared, by automated procedures, within 30 minutes. This fibrin sealant is completely autologous, essentially free from exogenous proteins and free from potentially harmful or infectious agents. It has excellent stability for at least 16 days when stored at -20°C (Hollingsbee DA & Edwardson, 1994). *In vivo* and *in vitro* fibrinolytic stabilises are similar to fibrin sealants which contain aprotinin, an anti-fibrinolytic protein (Cederholm-Williams, 1994). The preparation process is safe, reproducible, optimised and fully automated. It has been demonstrated to be effective for sealing air leaks, achieving haemostasis in low and high pressure systems, achieving microsurgical anastomosis and eliminating CSF fluid leakage (Edwardson *et al.*, 1994; Green *et al.*, 1994).

Unlike other fibrin sealants, batroxobin, a serine proteinase derived from snake venom is utilised to cleave fibrinogen and not thrombin. Clinical use of batroxobin is well documented, with over 10 years of clinical experience. It has a highly specific mode of action, cleaving only fibrinopeptide A from fibrinogen, which is particularly relevant to fibrin sealant kinetics, allowing greater control of sealant (fibrin II) formation. Unlike thrombin and other venom derived enzymes it does not activate platelets and retains its activity in the presence of heparin. Batroxobin poses no risk of infection and has a low intrinsic immunogenic potential. Hence batroxobin is a desirable, and potentially safe alternative to exogenous thrombin for use in fibrin sealants (Blomback *et al.*, 1994).

In the preparation of novel fibrin sealant, batroxobin is biotinylated, to facilitate its easy removal after it has completed it's function. The addition of avidin, immobilised to agarose, results in the binding of biotin to avidin, which is the strongest non-covalent interaction between a ligand and protein known in nature. The resulting avidin-agarose:biotin-batroxobin complex is then removed via filtration. The use of biotin and avidin in this process is both:

- safe - both biotin and avidin have excellent safety profiles, with highly purified pharmaceutical grades readily available and the avidin-agarose and biotin-batroxobin

formulations have been optimised to preserve activity, ensure stability and sterilised by gamma irradiation.

- effective - more than 99% of the biotin-batroxobin is removed within 5 minutes, with the formulation and yield of fibrin sealant unaffected (Bayer *et al.*, 1994).

Thus novel fibrin sealant overcomes problems which are associated with other fibrin sealants, namely infection and antigenicity.

1.4 Thesis Objectives

i. To obtain comprehensive quantitative data concerning physiological, structural, cellular and biochemical mechanisms during PSA development in a definitive experimental model.

ii. To obtain comprehensive quantitative data concerning physiological, structural, cellular and biochemical mechanisms during PSA prevention with novel fibrin sealant in a definitive experimental model.

iii. To identify modes of action in which novel fibrin sealant prevents PSAs.

iv. To assess PSA prevention by novel fibrin sealant using:

   a. different preparation and application techniques
   b. different animal species
   c. different PSA induction sites
   d. non-autologous and autologous systems.

v. To directly compare PSA prevention by novel fibrin sealant, Tissucol® and CRYO in a definitive experimental model.

2.0 Materials and Methods

2.1 Home Office Regulatory Guidelines

Scientific use of animals in the United Kingdom is carefully controlled by the UK Home Office, primarily through the enforcement of the Animal (Scientific Procedures) Act 1986. This act regulates "any experimental or other scientific procedure applied to a protected animal which may have the effect of causing that animal pain, suffering, distress or lasting harm".

The act is regulated though the issuing of licences, of which three are required for experimental work of this nature to be carried out:

i. Certificate of Designation - controls establishments where work is performed, with standards ensuring animal health and well being
ii. Project Licence - which determines the validity of the work and controls the level of severity which is acceptable
iii. Personal Licence - issued to people carrying out work specified under a project licence and guarantees that people are competent in their ability to perform such work.

Regulations of the Animal (Scientific Procedures) Act 1986 conform to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, Council of Europe) and achieve the standard of care required by the US Department of Health and Human Services' Guide for the Care and Use of Laboratory Animals.

2.2 Animal Models

Careful considerations need to be made when choosing an experimental model in which it is hoped data obtained will contribute to clinical understanding. These considerations can be grouped into four main categories:

i. clinical relevance for fair and direct comparisons between experimental and clinical

work

ii. an accepted model for good reception of the work by the scientific community

iii. valid and accurate model

iv. availability, housing and cost of the model.

The majority of experimental procedures carried in the UK are on mammals, due to their physiological similarities to humans, with the species chosen being the lowest on the phytogenetic scale which is likely to satisfy the aims of the study.

2.2.1 Rabbit Uterine Horn Abrasion Model

Rabbits are one of the most commonly used experimental models, with main breeds utilised being New Zealand White, Dutch and Half Lop. Their extensive use is due mainly to their ease of availability, housing and relatively low cost.

Rabbit models have been used extensively for studies investigating PSAs, many making use of the uterine horns as experimental sites (for example, Neuwirth and Khalaf, 1975; Nishimura *et al.*, 1983; Diamond *et al.*, 1987; De Laco *et al.*, 1994; Marana *et al.*, 1997; Rodgers *et al.*, 1997; Takeuchi *et al.*, 1997; De Laco *et al.*, 1998). These studies have devised various methods for induction of PSAs, ranging from the introduction of foreign materials to mechanical injuries such as scalpel incisions and crushing injury, with many different potential PSA prevention agents tried, ranging from fibrin sealants (Takeuchi *et al.*, 1997, De Laco *et al.*, 1994) to anti-inflammatory peptides (Rodgers *et al.*, 1997).

As detailed in the introduction to this thesis, a scientific failing with previously conducted studies is their qualitative nature. All rabbit studies reported in this thesis employed a definitive experimental model and quantitative analytical procedures. The model used was a slightly modified version of a widely used experimental system for the testing of potential peritoneal PSA preventative agents and has been optimised over several studies prior to these investigations. In summary a standard abrasion injury was inflicted on each uterine horn and ipsilateral peritoneal wall, which would naturally lie in juxtaposition.

The injured areas were then held apposed using positional sutures, placed outside the experimental site, with treated sites separated by a layer of fibrin sealant. Experimental sites were then left *in vivo* for the length of recovery time dictated by the study protocol. Due to the level of injury induced and injured surfaces being apposed this rabbit uterine horn abrasion model is a severe experimental PSA model, with a "worse case" scenario created.

To ensure that injuries of consistent standard area could be induced, templates, 25 mm x 3mm, designed to fit the uterine horn or peritoneal wall, were specially constructed. Abrasions were induced using a scraper with a standard depth (1.5mm) which fitted the exact area of the template. With the additional parameter of using a set number of scrapes for each injury site, injuries were maximally standardised within the limits of biological variation.

Quantitative analysis was carried out for all studies with stereological techniques employed for this purpose due to their precise and unbiased nature (refer to section 2.14). This becomes especially important when comparing control and treated cases, as without such rigid regulations governing analytic procedures biases can be easily introduced.

Uterine horns were chosen as the experimental site, together with the peritoneal wall, due to their size, structure and location within the peritoneal cavity. Heavy handling of tissues has been shown to induce PSA formation (Boys, 1942; Connolly & Smith, 1960) and hence excessive tissue handling needed to be avoided. In addition, ex-breeding rabbits were also chosen for these investigations due to their large size facilitating appropriate procedures.

2.2.2 Pig Colonic, Caecal and Stomach Abrasion Model

Pigs are one of the usual experimental models chosen when a larger model is required. Significantly less published information is available detailing the use of porcine models

with regard to PSAs. This is most probably due to the increased cost of using large animals in experimental work, both with regard to housing and surgical procedures, and with smaller models being acceptable for most PSA work.

Porcine models previously used to investigate PSAs (Fowler *et al.*, 1994; Reissman *et al.*, 1996; Christoforoni *et al.*, 1996) demonstrate, unsurprisingly, that pigs form PSAs in response to injury or trauma by identical pathogenesis to humans. Some success has been shown with preventative agents although their absolute value has as yet to be substantiated due to the limited number of published studies.

Using knowledge obtained through the use of the rabbit uterine horn abrasion model, the pig abrasion model was also optimised and standardised. As with the rabbit model, templates were specially constructed, 25mm x 3mm, to fit tissue areas and were used in conjunction with a scraper of known depth, 1.5mm. A set number of scrapes for each injury site was again employed and therefore, the injury was maximally standardised within the limits of biological variation. Additional use of quantitative analytical procedures ensured unbiased and precise results.

One of the main reasons that a pig model was chosen for these studies was due to its large size and more importantly the larger volume of blood which could be taken. This allowed for truly autologous novel fibrin sealant to be made and tested. That is blood was collected from an anaesthetised animal prior to the surgical procedure, novel fibrin sealant was produced from this blood and then used to treat abrasion injuries in that animal.

2.3 Animal Husbandry

2.3.1 New Zealand White Rabbits

Female, New Zealand White, ex-breeding rabbits, approximately 3.5kg in weight were supplied from Goreside Rabbits, UK or from Harlan Rabbits, UK, approved Home Office sources.

Rabbits were admitted to the department at least one week prior to commencement of the study to allow re-acclimatisation and full clinical observation to be made. All animals were examined and monitored for disease or injury before being admitted to the study and randomised to one of the control or treatment groups. Animals were individually labelled and housed singly in racked cages with grid floors. The environment was monitored for humidity and temperature. Food and water were allowed *ad libitum* until 18 hours prior to surgery, when food was withdrawn.

2.3.2 White Landrace Cross Pigs

Large white landrace cross pigs, 15-25kg in weight, used in these studies were obtained from Fulmer Place Farm, an approved Home Office source. All animals were brought in to the department at least one week prior to surgery for acclimatisation and clinical observations, with only healthy animals admitted to the study, randomly assigned to a control or treatment group. Animals were individually identified by an ear tag and housed singly in pens with sawdust bedding. The environment was monitored for humidity and temperature. Following standard procedures, food and water were allowed *ad libitum* until 18 hours prior to surgery, when food was withdrawn.

2.4 Study Groups

2.4.1 Rabbit

2.4.1.1 Pathogenesis of PSAs

Each experimental group consisted of 4 animals, with each animal injured at two sites, giving 8 experimental sites per group. In order to gain an accurate picture of the dynamics of PSA development, tissues were sampled at specified time points following injury; 30 seconds, 1, 3, 5, 10, 30 minutes, 1, 4, 8, 16 hours, 1, 3, 5, 7, 17, 28 and 42 days.

2.4.1.2 Prevention of PSAs with Vivostat™ System Human Derived Fibrin Sealant

In order to understand the mechanisms responsible for the reduction and/or prevention of PSAs using Vivostat™ system human derived fibrin sealant (novel fibrin sealant) a similar study to that investigating pathogenesis of PSAs (control situation) was performed.

Surgical and analytical procedures were completed on the Pathogenesis of PSAs study before the novel fibrin sealant study was commenced. Analysis of control data revealed that tenacious PSAs were evident at 1 hour post injury and all subsequent time points. Hence experimental investigation of novel fibrin sealant treated groups was started at 1 hour, with further sampling at 4, 8, 16 hours, 1, 3, 5, 7, 14, 28 and 42 days. Each sample time point consisted of 4 animals, two experimental sites per animals, hence 8 experimental sites per group.

2.4.1.3 Comparison of Preparation and Application Methods of Vivostat™ System Human Derived Fibrin Sealant for PSA Prevention

This study, consisting of 5 experimental groups, investigated various novel fibrin sealant application methods with regard to their efficiency to prevent or reduce PSAs. Animals, all 14 day recovery, were randomised to one of the following groups:

Treatment Group	Vivostat™ Application Method	Number of Animals	Number of Experimental Sites
Control	-	7	13
Device Prepared Novel Fibrin Sealant	Spray	6	12
Device Prepared Novel Fibrin Sealant	UNES Gun	6	12
Device Prepared Novel Fibrin Sealant	Needle	6	12
Bench Prepared Novel Fibrin Sealant	UNES Gun	6	12

2.4.1.4 Comparison of Vivostat™ System Human Derived Fibrin Sealant, Tissucol® and CRYO for PSA Prevention

Three groups compared different fibrin sealants for their PSA prevention or reduction capacities together with a control group. Animals, 14 day recovery, were randomised to one of the following groups:

Treatment Group	Number of	
	Animals	Experimental Sites
Control	Control cases were taken from Application Comparison Study	
Device Prepared Novel Fibrin Sealant (Needle Application)	6	12
Tissucol® - 4 Units Bovine Thrombin - 500 Units Bovine Thrombin	6	 5 6
CRYO - 4 Units Bovine Thrombin - 500 Units Bovine Thrombin	6	 6 6

2.4.2 Pig

2.4.2.1 Pathogenesis of PSAs and Prevention with Vivostat™ System Human and Porcine Derived Fibrin Sealant in the Pig Abrasion Model - Pilot Study

This study consisted of 3 experimental groups investigated PSA prevention or reduction using human blood derived and porcine blood derived novel fibrin sealant together with a control group in the pig abrasion model. Details of each group, all 7 day recoveries, were as follows:

Treatment Group	Number of Animals	Injury Site	Number of Experimental Sites
Control	3		Total : 9
		Stomach	3
		Colon	4
		Caecum	2
Human derived novel fibrin sealant	3		Total: 8
		Stomach	2
		Colon	4
		Caecum	2
Porcine derived novel fibrin sealant	2		Total: 5
		Stomach	1
		Colon	2
		Caecum	2

Porcine novel fibrin sealant group contained one post operative mortality, where the cause of death was a strangulated hernia due to suture breakdown.

2.4.2.2 Pathogenesis of PSAs and Prevention with Vivostat™ System Human Derived Fibrin Sealant in the Pig Abrasion Model - Main Study

The purpose of this study was to substantiate and expand on the results obtained from the pilot study. This main study consisted of control and human blood derived novel fibrin sealant treated groups, with animals recovered after 14 days.

Treatment Group	Number of Animals	Injury Site	Number of Experimental Sites
Control	5		Total : 10
		Stomach	5
		Colon	5
Novel fibrin sealant	6		Total: 12
		Stomach	6
		Colon	6

One control animal was a post operative mortality due again to a strangulated hernia attributed to suture breakdown.

2.5 Surgical Procedure

Surgical procedures for studies investigating pathogenesis of PSAs and prevention with novel fibrin sealant studies were carried out by Miss SA Ricketts and Dr PD Sibbons. For all other studies surgical procedures were carried out by Dr PD Sibbons.

2.5.1 Rabbit

Premedication was administrated with hypnorm (Fentanyl citrate 0.315mg/ml and fluanisone 10mg/ml. Supplied by Janssen Saunderton, High Wycombe, Buckinghamshire.) (0.2ml/kg body weight intramuscular) to the right gluteous maximus muscle.

Surgical anaesthesia was induced with hypnorm (0.3ml/kg body weight intramuscular) and diazepam (5mg/ml diazepam. Supplied by Phoenix Pharmaceuticals Limited, Gloucester.) (2.5mg/kg body weight intravenous). Full sterile operating procedures were observed. Particular attention was paid to the washing of surgical gloves in sterile water to remove all traces of particulate matter (starch, powder) which might, if transferred to the operative site, induce granuloma or adhesion formation.

Fur was shaved from the surgical area and the area scrubbed with alcoholic chlorhexidine followed by iodine. Laparotomy was performed using cutting diathermy, by a single incision in the midline, from lower liver margin to the level of the iliac fossa, through skin and muscle to peritoneum, which was divided with scissors. Retraction was applied to laparotomy wound edges to allow access to uterine horns (Figure 2.1).

Experimental areas were selected on the serosal surface of each uterine horn and corresponding internal ipsilateral surfaces of the peritoneum, which would naturally lie in juxtaposition with each other. A standard template, constructed specifically either for the uterine horn or peritoneal wall, was placed on the selected areas (Figures 2.2 and 2.3), and each area abraded with the sharp edge of a scraping tool (Figure 2.4). A standard number of 10 scrapes was used to caused homogeneous punctate bleeding but not so far

as to cause frank contiguous haemorrhage (Figures 2.5 and 2.6). Hence a standard, and reproducible injury was produced in each case.

Single throws of Stannius positional suture (Ethibond 6/0 or similar) were placed 5mm outside each end of abraded areas (peritoneal wall and uterine horn) (Figure 2.7). These sutures passed only through the serosa of the peritoneum and corresponding uterine horn. For control groups, sutures were tightened to bring the two juxtapositional abraded areas together and maintain contact between the two areas. Timing was commenced from this point. For treated groups positional sutures were tightened to bring the two abraded areas close together but not in contact and fibrin sealant was then applied (refer to Chapters 4, 6 and 7 for volumes) to abraded areas (Figure 2.8). Sutures were then drawn together to bring the two areas into contact, using as much tension as was needed to create reasonable contact but avoiding tight sutures. Timing commenced from this point.

In groups which sampled up to 30 minutes post injury, rabbits were kept anaesthetised, in the supine position with the laparotomy incision held closed with tissue clamps. In groups which sampled from 1 hour post injury onwards, the laparotomy was closed and reopened after the relevant time period.

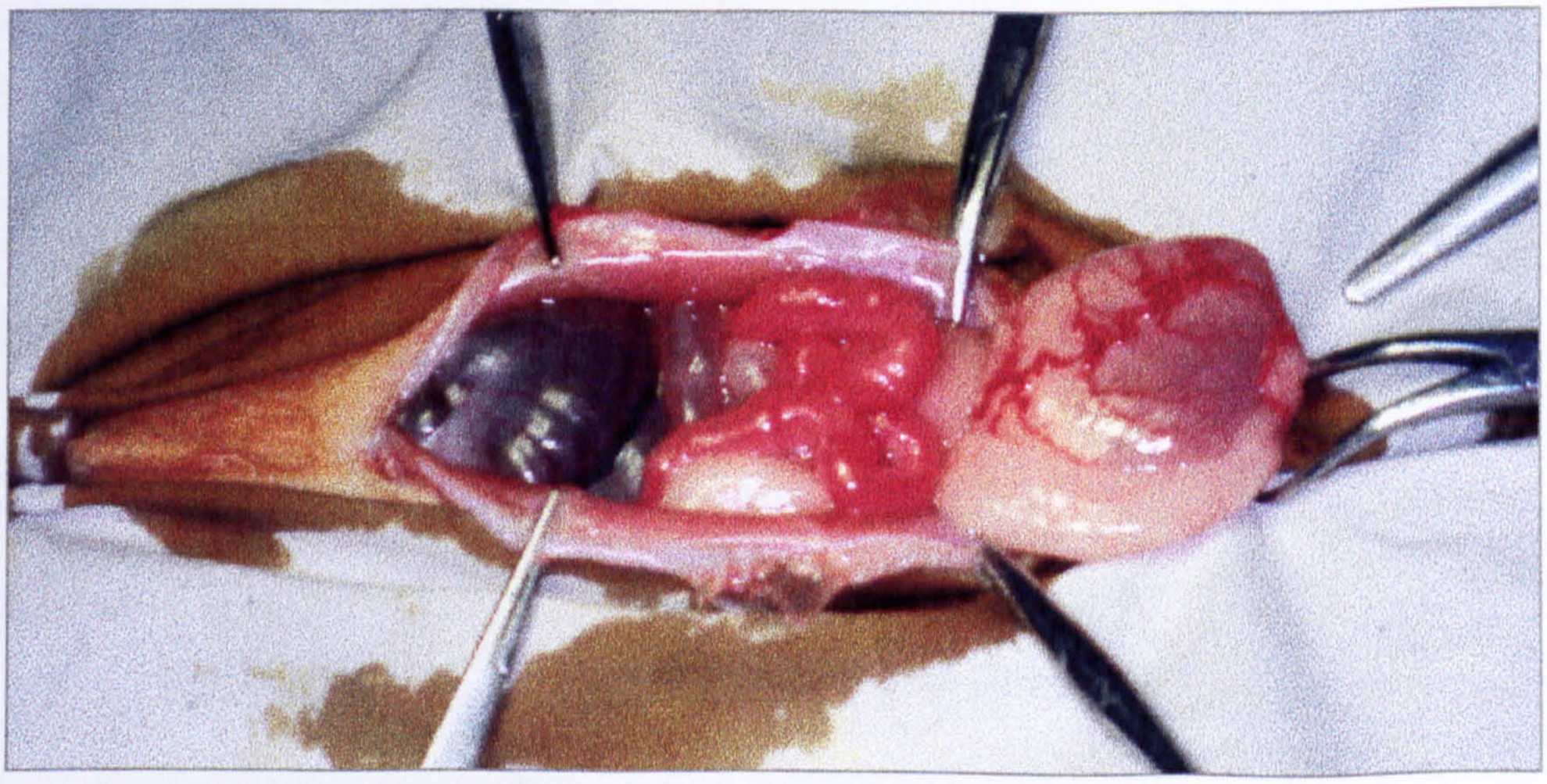


Figure 2.1: Laparotomy opening and retraction of wound edges to show location of uterine horns.

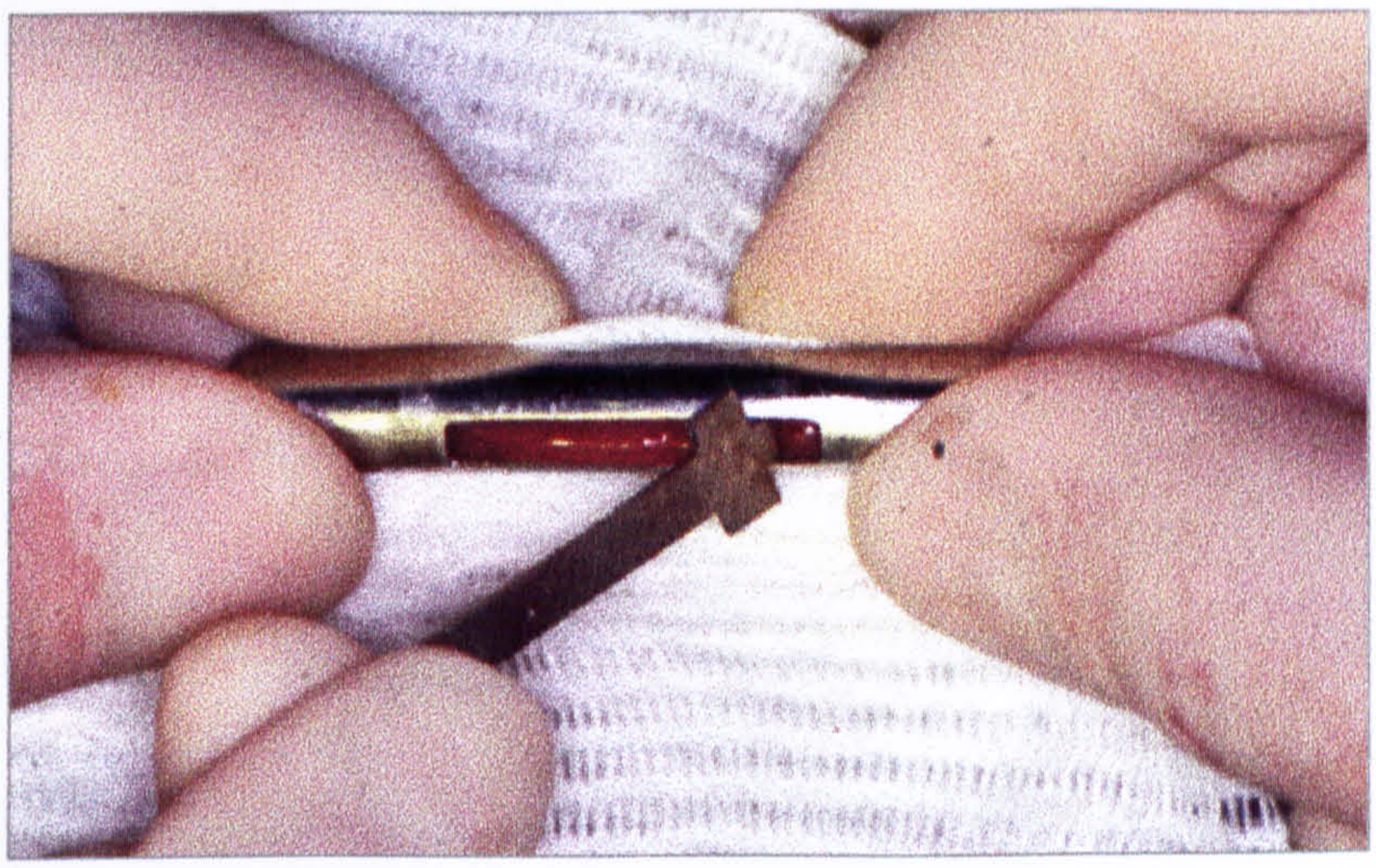


Figure 2.2: Uterine horn template on selected area of uterine horn.

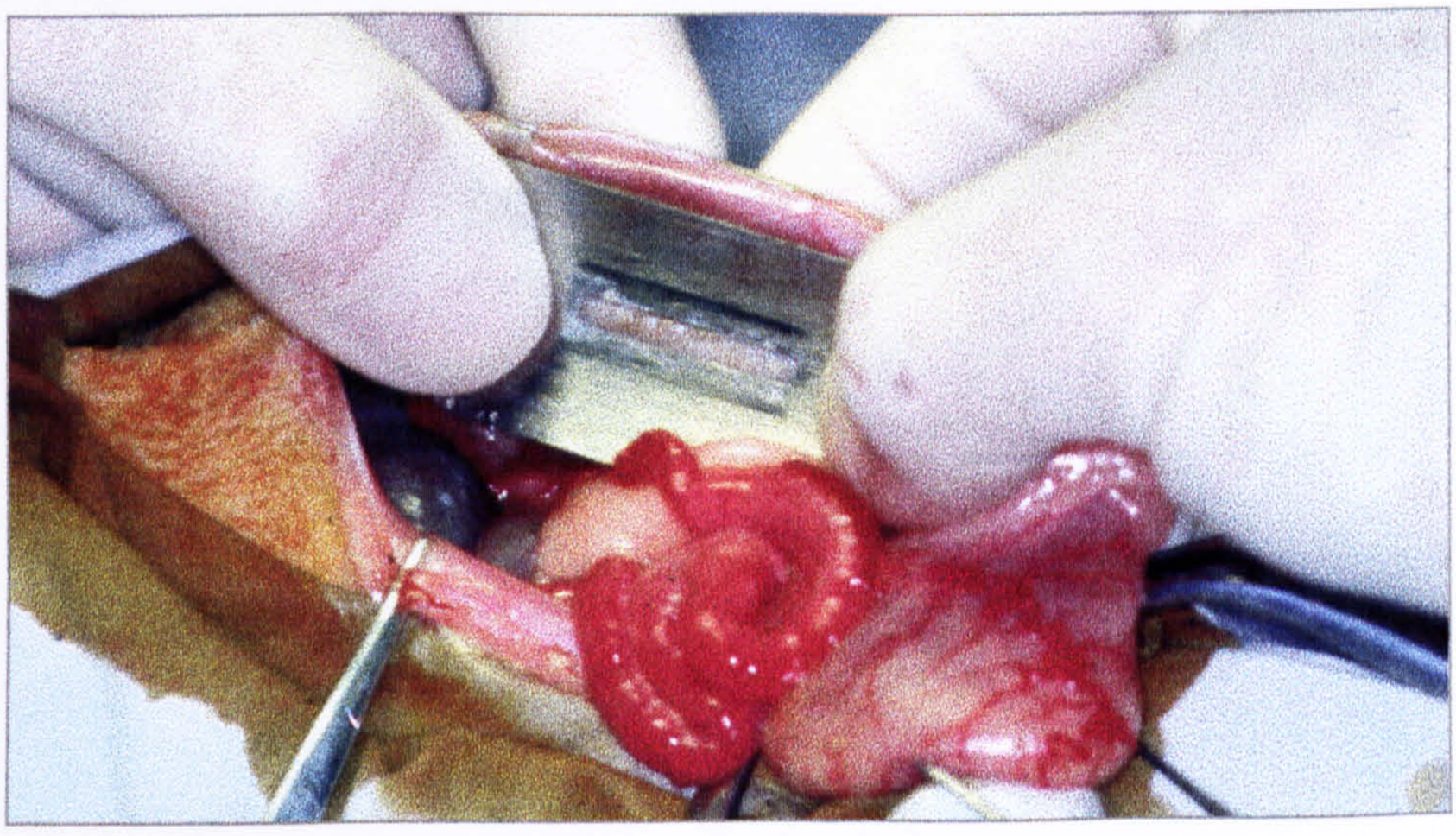


Figure 2.3: Peritoneal wall template on selected area of peritoneal wall.

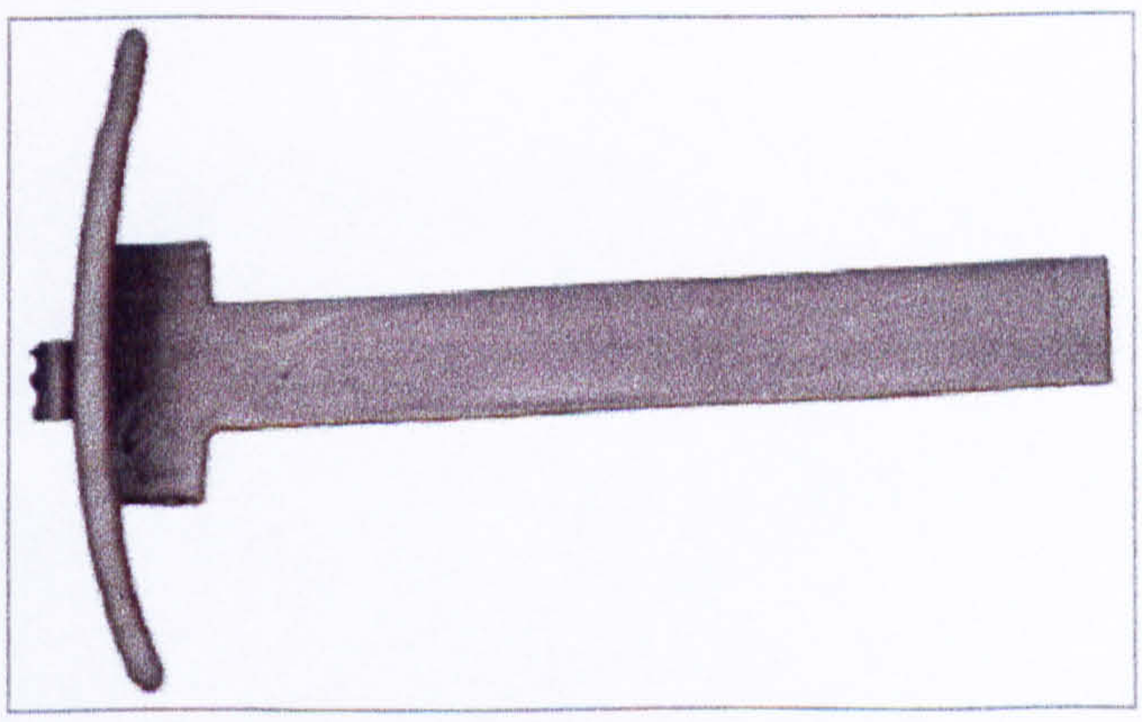


Figure 2.4: Scraping tool used to abrade tissues.

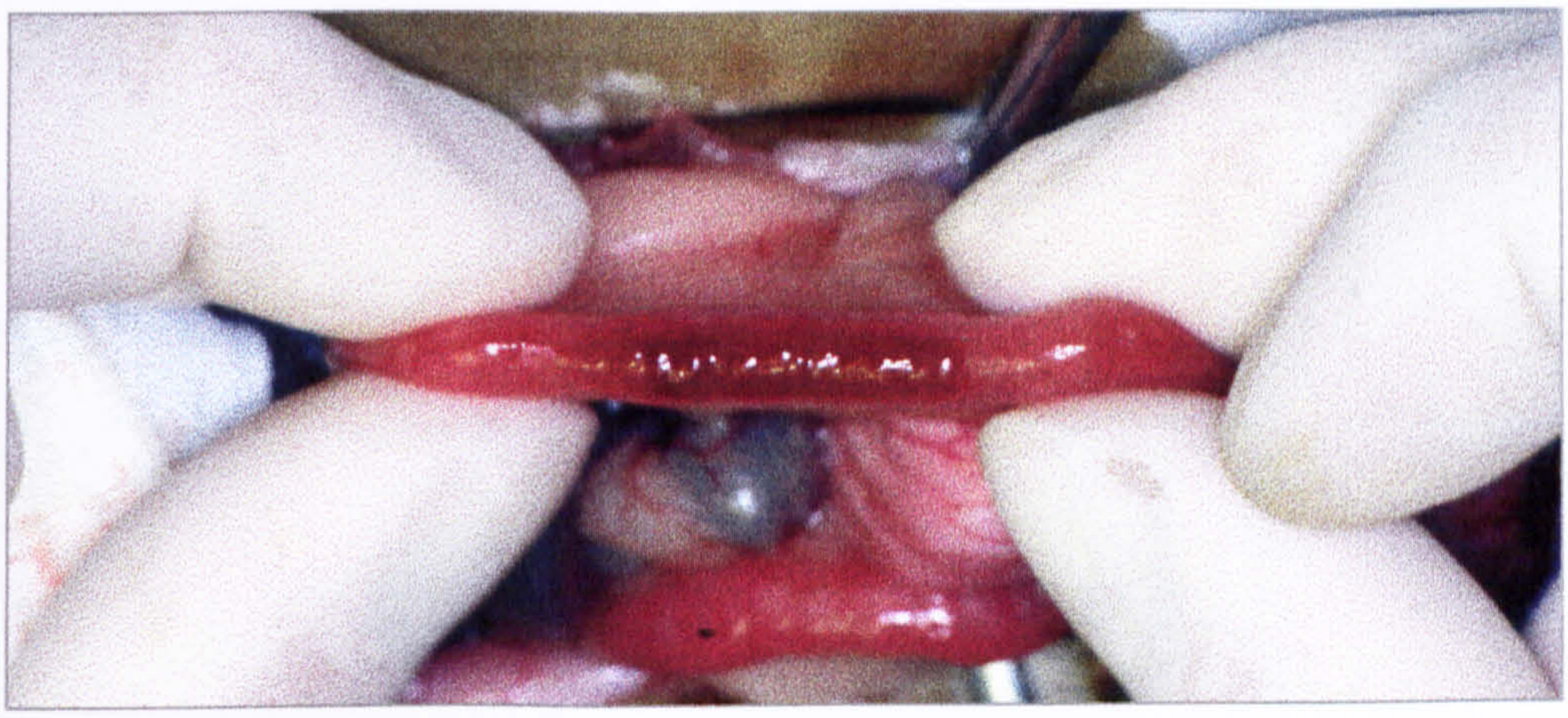


Figure 2.5: Standard and reproducible abrasion injury on a uterine horn.

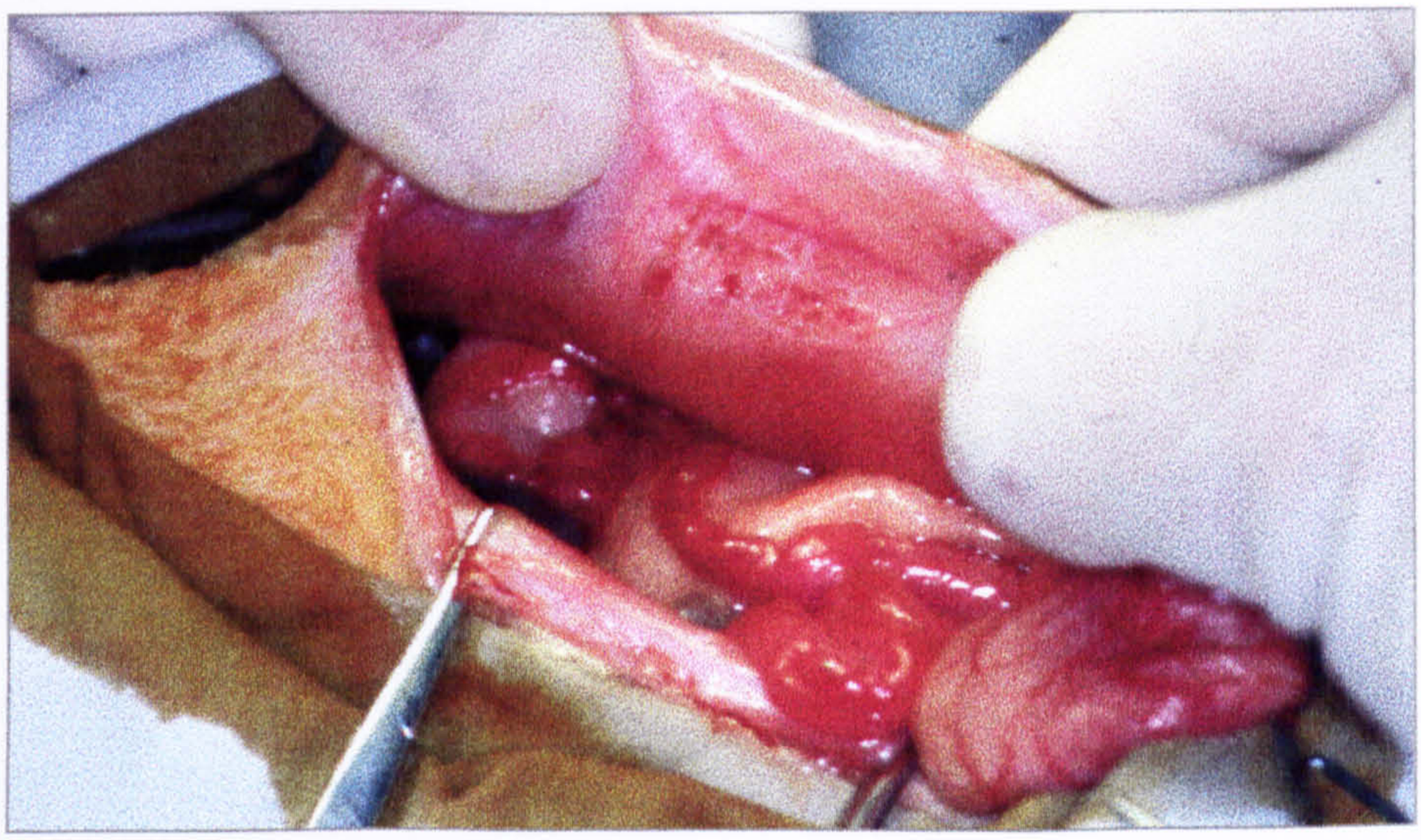


Figure 2.6: Standard and reproducible abrasion injury on the peritoneal wall.

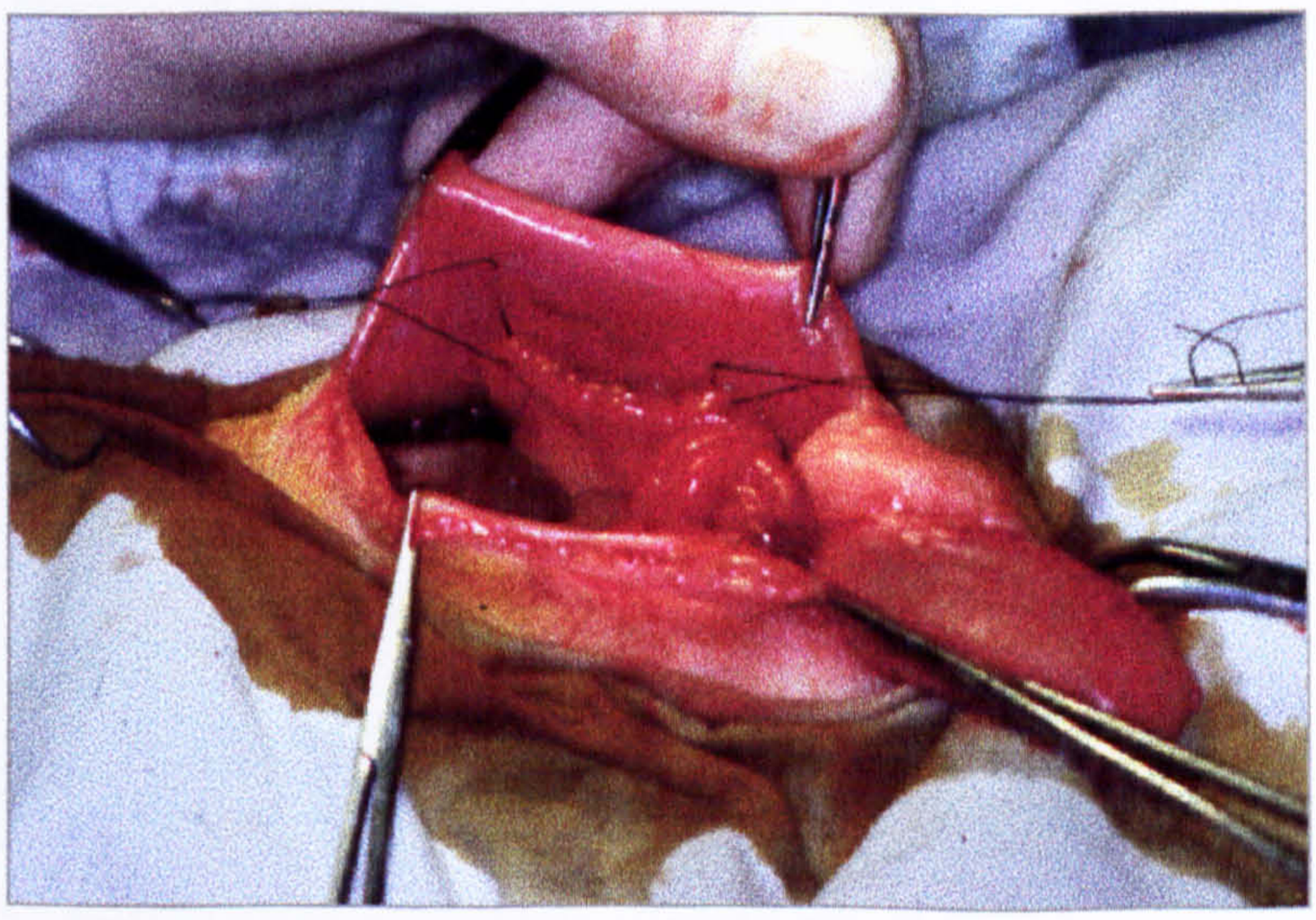


Figure 2.7: Positional sutures placed outside the experimental site.

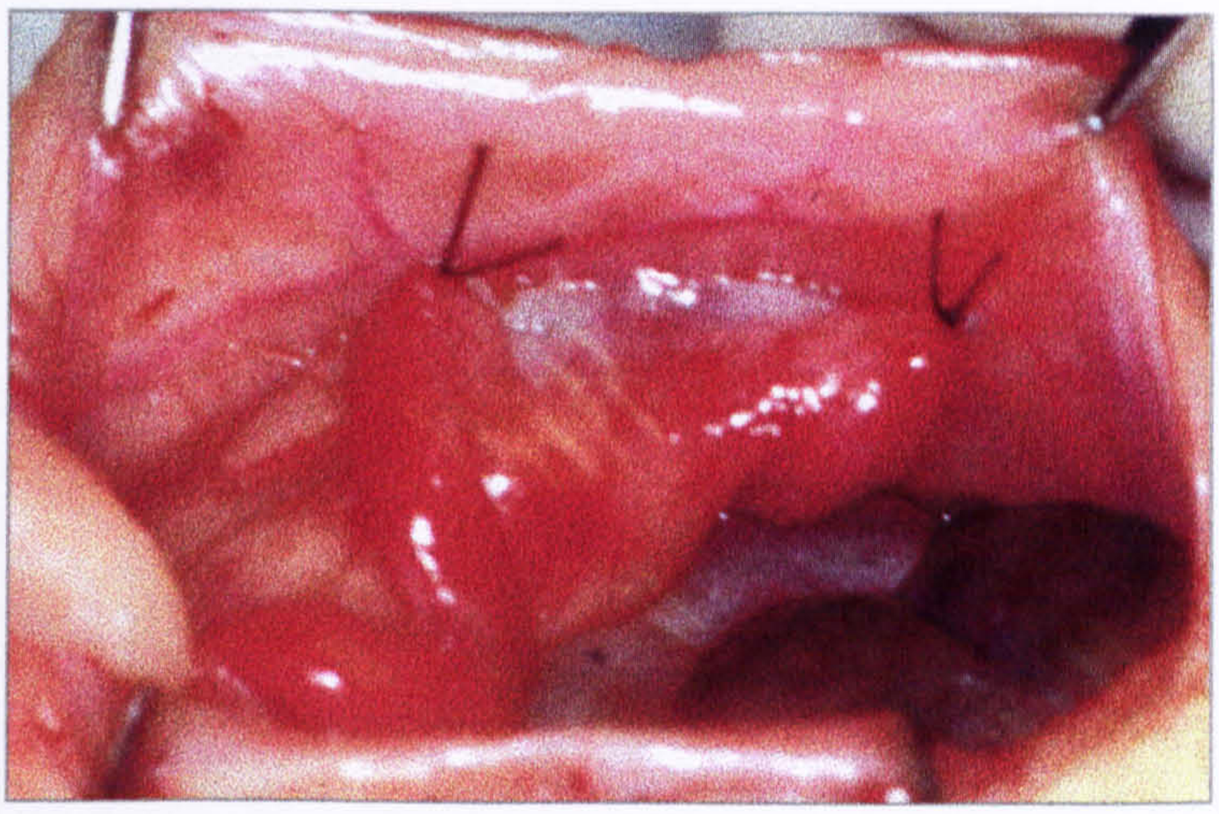


Figure 2.8: Fibrin sealant applied to injured surfaces.

Laparotomy wound closure was in two layers: first with 2/0 plain catgut on atraumatic half round needle for the peritoneum and muscle layers, using transplant longflow, over-under, non-interrupted crossed sutures; and secondly with 2/0 Prolene or similar on half round cutting needle using interrupted mattress sutures for the skin. Immediately after closure of the wound, "Opsite" (Smith and Nephew Medical Limited, Hull) wound dressing was applied on and around the wound to create "second skin" barrier.

Rabbits were allowed to recover from anaesthesia in a warm, quiet environment. Once fully recovered, rabbits were removed to normal accommodation and allowed food and water *ad libitum*. All rabbits were subsequently monitored for possible procedure associated sequelae, for example, wound infection, wound leakage, temperature elevation, diarrhoea, constipation, appetite decrease or immobility.

2.5.2 Pig

Pre-medication was administered using Ketamine (Ketalar-50mg/ml ketamine hydrochloride. Supplied by Parke-Davis, Pontypool, Gwent.) (5mgs/Kg) plus Xylazine (Rompun 2%-Xylazine hydrochloride 23.32mg/ml (equivalent to 20mg/ml xylazine) and 1mg/ml methyl 4-hydroxy-benzoate (preservative). Supplied by Bayer Plc., Animal Health Business Group, Bury St. Edmunds, Suffolk.) (1mg/kg)) intramuscularly in the gluteous maximus muscle. At the operating suite, pigs were induced to and maintained at full anaesthesia with Halothane at 4% in oxygen and nitrous oxide delivered at 1.5 litres

per minute and 0.5 litres respectively per minute via mask to the snout.

Once anaesthetised pigs were transferred to lay in the supine position on the operating table, where they were secured by soft tapes to each limb. A plastic ear tag bearing the unique pig identification number for the study was secured to one of the animals ears. For each pig, the surgical site was shaved and scrubbed with chlorhexidine in alcohol followed by swabbing twice with iodine in alcohol. Sterile operative procedures were adhered to from this point on.

The animals were draped. Cutting diathermy and scissors were used to expose the spiral colon and the stomach through a midline laparotomy starting at the level of the distal xyphoid process of the sternum and extending distally 10 - 12cms.

One area was selected on the lateral surface of the spiral colon or on the blunt end of the caecum and the medio-lateral serosal aspect of the stomach such that these areas each laid naturally against the ipsilateral peritoneal wall. Areas were then selected on the ipsilateral peritoneal wall. As with the rabbit uterine horn abrasion model, each selected area was abraded, using a template and scraper (Figures 2.9 and 2.10), a standardised number of 12 scrapes which caused homogeneous punctate bleeding but not so far as to cause frank contiguous haemorrhage was performed (Figures 2.11 and 2.12).

A single throw suture (Ethibond 0.6 or similar) was placed at each end of the experimental site but outside of the abraded areas (Figure 2.13). These sutures passed only through the internal serosa of the peritoneum and then passed through the serosa at the respective ends of the apposed abraded areas on the stomach or colon. Sutures were tightened to bring the two apposed abraded areas close together but not in contact. For treated cases, human or porcine novel fibrin sealant was applied to the abraded areas (mean volume of 1.3ml per experimental site) (Figure 2.14), whereas no treatment was applied in control cases. Positional sutures were then tightened to bring the two abraded areas into close contact.

The laparotomy was closed in two layers, first with 2/0 Dexon Plus on an atraumatic half round needle for peritoneum and muscle layers, second with 2/0 Prolene or similar on half round cutting needle for skin. The first suture layer was of "transplant" longflow, over-under, non-interrupted crossed sutures, the second layer was of interrupted cruciate mattress sutures. Immediately after closure of the skin wound, "Opsite" (Smith and Nephew Medical Limited, Hull) was sprayed on and around the wound to create "second skin" barrier.

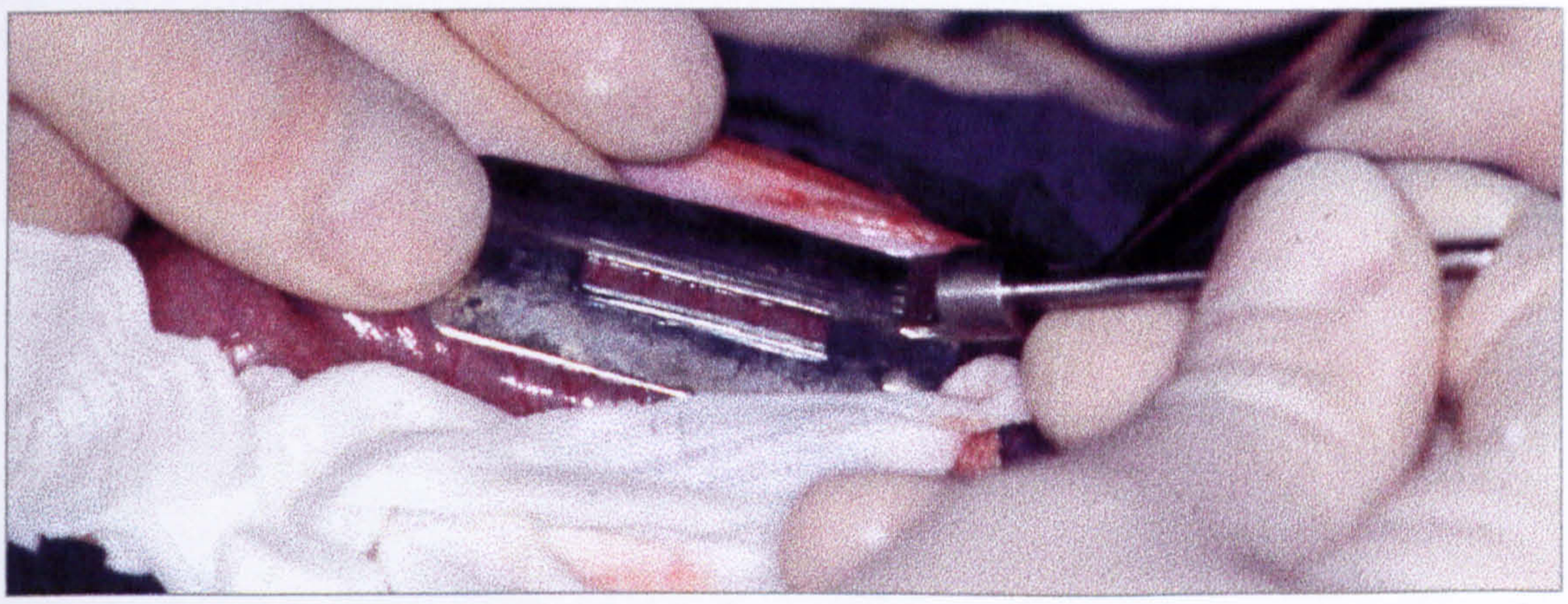


Figure 2.9: Selected area of caecum during abrasion using the template and scrapper.

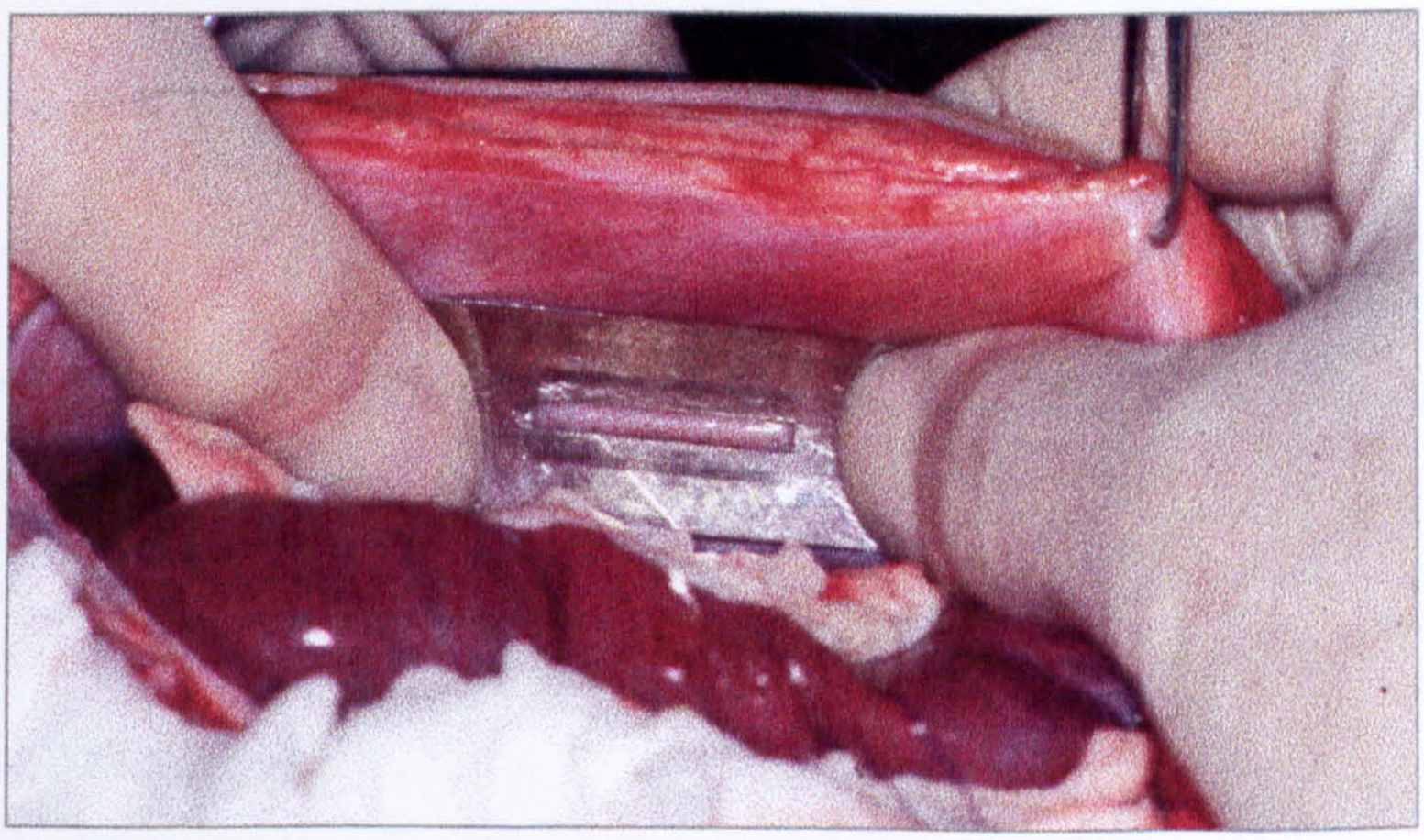


Figure 2.10: Peritoneal wall template in position prior to abrasion.

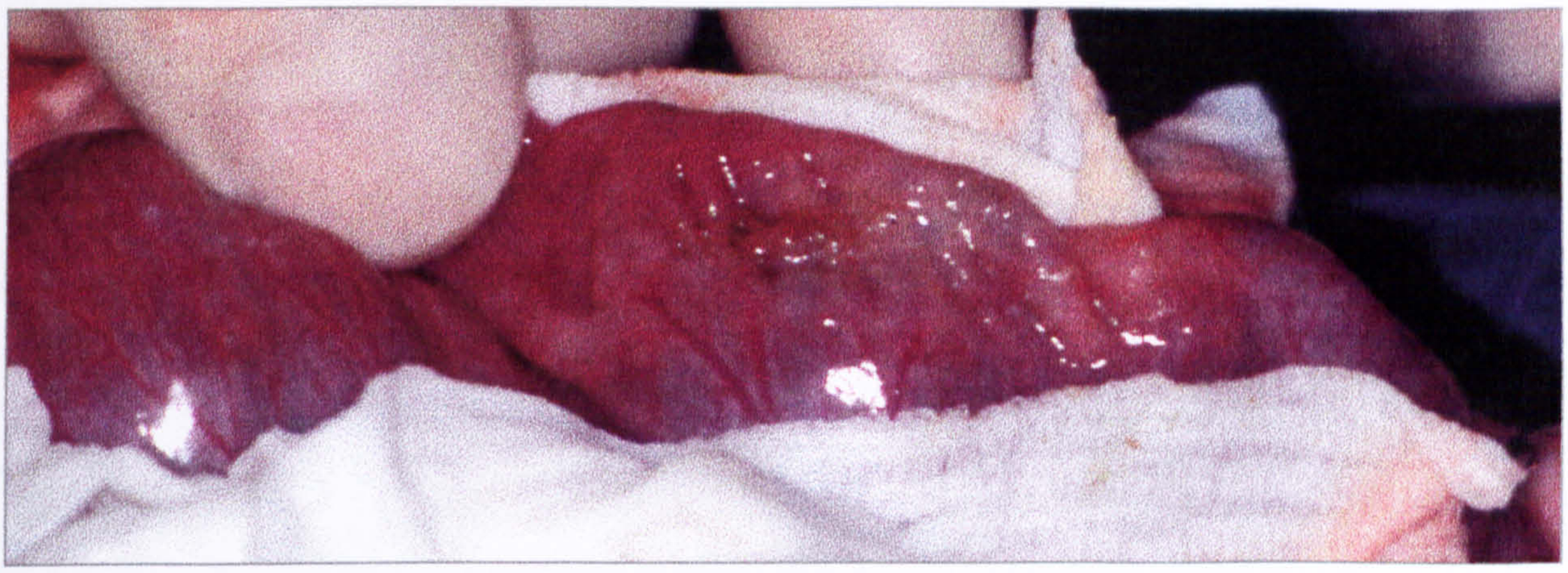


Figure 2.11: Standard and reproducible abrasion injury on the caecum.

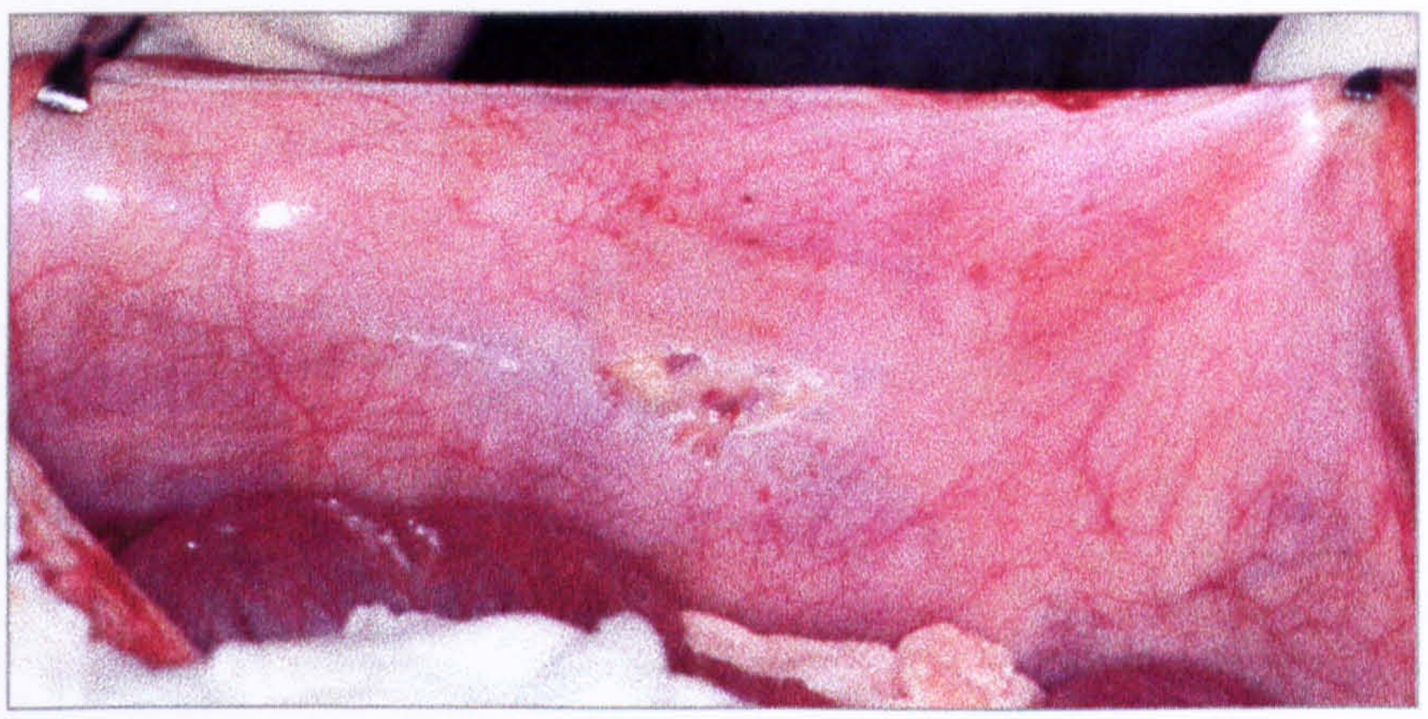


Figure 2.12: Standard and reproducible abrasion injury on the peritoneal wall.

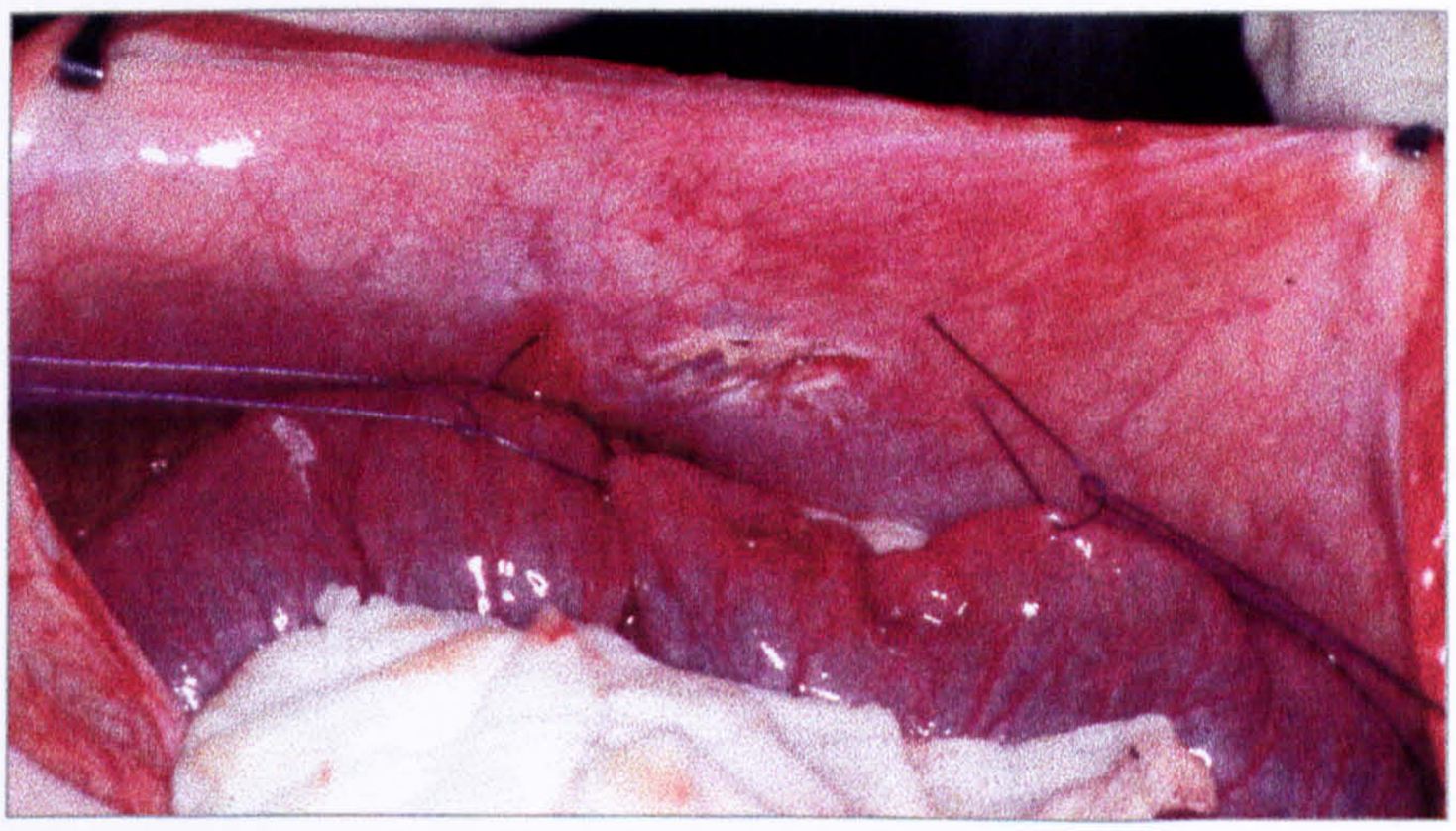


Figure 2.13: Positional sutures thrown outside the experimental site.

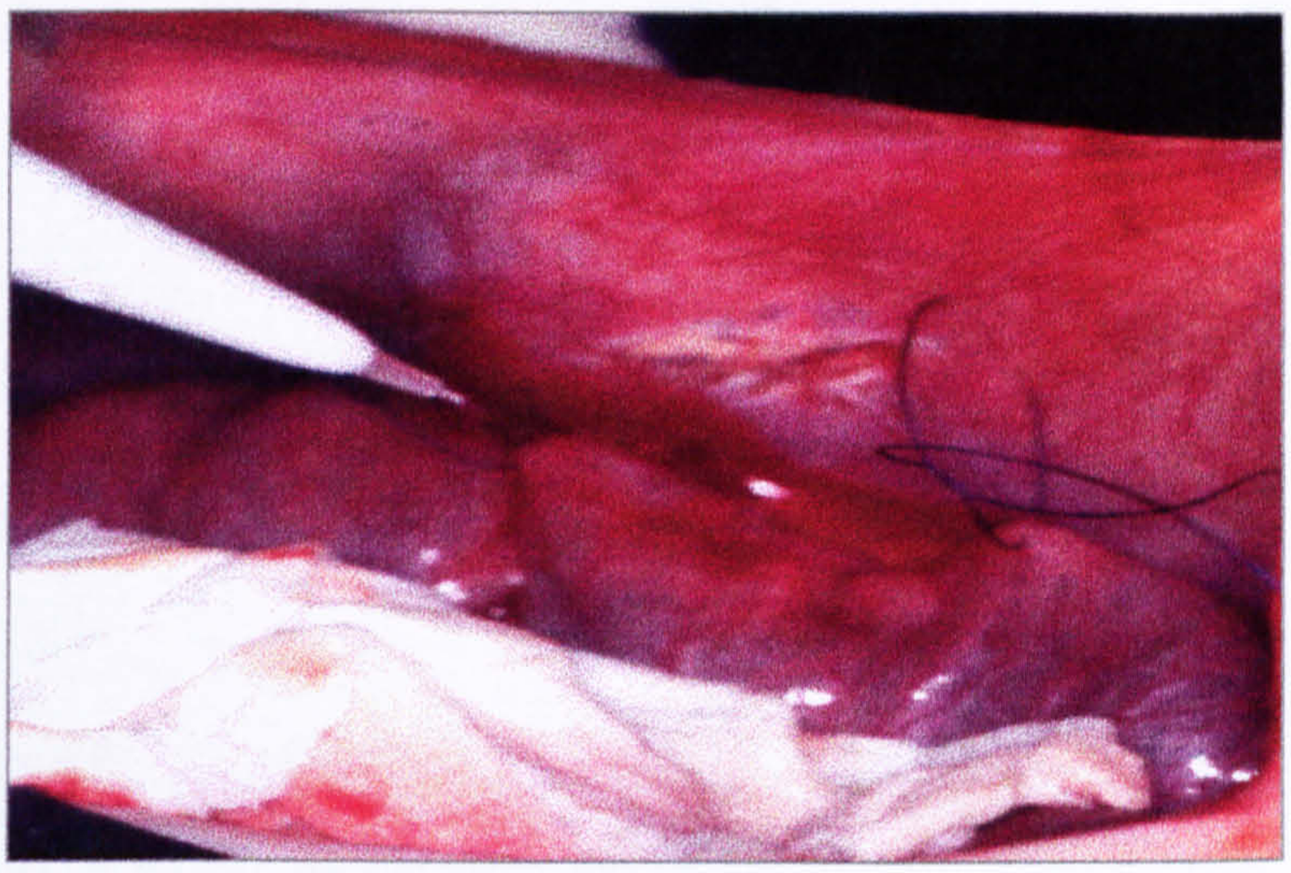


Figure 2.14: Novel fibrin sealant application to the experimental site.

Pigs were allowed to recover from anaesthesia in a recovery area. Proprietary analgesics (Temgesic (Buprenorphine 0.3mg/ml as hydrochloride. Supplied by Reckitt & Coleman Products Ltd., Hull.) (5mgs intramuscular)) were administered. Once fully recovered, animals were returned to normal accommodation with twice daily feeds and water *ad libitum*. For the pilot study, pigs were monitored and recovered for 7 days and for the main study for 14 days.

2.6 Termination Procedure

Animals were anaesthetised as for the surgical procedure. Laparotomy was reopened along the original incision, with experimental areas identified and macroscopic observations recorded and photographed. The abraded area of the peritoneum together with a border of unabraded tissue in excess of 5mm was resected. The associated uterine horn in the rabbit studies, attached to the peritoneum by positional sutures at both ends of the abraded area, was resected away from the omentum and remaining uterine horn. For the pig studies, the colon, caecum and stomach experimental sites, joined to the peritoneum by positional sutures at each end of the site, were resected. Euthanasia was achieved by high dose intravenous pentabarbitone (Expiral-Pentobarbitone sodium BP 200mg/ml. Supplied by Sanofi Animal Health Ltd., Watford, Hertfordshire.) (150mg/kg body weight)).

Resected tissues were trimmed of excess adipose tissue, pinned flat on stiff card, to retain a standard, lifelike tissue positional relationship, and immersion fixed in 10% neutral buffered formal saline for at least 24 hours at room temperature.

2.7 Blood and Peritoneal Fluid Sample Collection

Blood and peritoneal fluid samples were collected at specific times throughout the surgical and termination procedures for studies investigating pathogenesis of PSAs and prevention with novel fibrin sealant. Blood samples were taken prior to the surgical procedure to act as intra-case controls (blood sample 1) and blood and peritoneal fluid samples were taken immediately following initial laparotomy (blood sample 2 and peritoneal fluid sample 1) and following the second (termination) laparotomy (blood sample 3 and peritoneal fluid sample 2).

Blood samples were taken from a peripheral vein (external jugular or bracheo-cephalic vein), placed into labelled EDTA tubes and gently mixed to ensure complete anticoagulation. Wedge blood films were made, a sample taken for analysis by Coulter Counter with the remaining sample then centrifuged at 1000xg for 10 minutes to obtain plasma which was quick frozen and stored at -80°C.

Peritoneal fluid samples were taken either undiluted directly from the peritoneal cavity or via a 5ml saline wash when insufficient undiluted fluid was present (dilution to be subsequently calculated via a protein assay). Samples were treated in the same manner as blood, being placed into EDTA tubes, gently mixed and subsequently stored at -80°C.

2.8 Prevention of PSAs using Fibrin Sealants

2.8.1 Vivostat™ System Human Derived Fibrin Sealant

The Vivostat™ system is a medical device for the peri-operative preparation and intra-operative application of a completely autologous human fibrin sealant that does not require the addition of exogenous thrombin.

The Vivostat™ system incorporates four integral components:

i. An automated processor unit - a compact, non-sterile, reusable, fully automated electro-mechanical device that controls and drives the biochemical process that is housed within the disposable preparation unit. The processor incorporates innovative technology that automatically separates fibrin from whole blood and prepares a sterile concentrated fibrin I solution.

ii. A disposable preparation unit - a sterile, closed, single-use, disposable device with integral and auxiliary reagents, that collects the blood, houses the biochemical process and harvests the resultant fibrin.

iii. An automated applicator unit - a compact, non-sterile, reusable, electro-mechanical device which houses the resultant fibrin and pH10 buffer cartridges and feeds the applicator set.

iv. A disposable applicator set - a sterile, single-use, disposable surgical instrument incorporating a non-blocking, adjustable spray head. The "fibrin-pen" connects to the applicator unit using an ultra-thin, disposable tube.

In addition to the preparation of human novel fibrin sealant autologous porcine novel fibrin sealant can be successfully prepared via this automated system.

2.8.1.1 Device Preparation

The recovery of fibrin I from whole blood was performed in the fully automated microprocessor controlled device under sterile conditions in less than 30 minutes. The device incorporates in-line process controls that ensure reproducibility and includes a state-of-the-art surgical instrument for controlled application of the resulting autologous fibrin sealant.

Freshly drawn anti coagulated whole blood (120ml plus 17ml 4% trisodium citrate USP) is separated and the resulting plasma (60ml) reacted with biotin-batroxobin for 10 minutes at 37°C. The acid soluble fibrin I polymer produced is isolated by centrifugation

and dissolved in 3.5 - 5.2ml 0.2M sodium acetate buffer (pH4) containing calcium ions.

The result is 4 to 6ml of concentrated fibrin I (20 ± 2mg/ml) which is stable for several days at -20°C. Trace amounts of biotin-batroxobin are removed by addition of freeze-dried avidin covalently coupled to agarose, which hydrates the fibrin I. Within 5 minutes the biotin-batroxobin:avidin-agarose complex is removed by filtration and the purified, concentrated fibrin I is transferred to the applicator device (Figure 1.13).

2.8.1.2 Bench Preparation

Freshly drawn venous blood was collected into two, 50ml centrifuge tubes, each containing 7ml 4% tri-sodium citrate buffer, to a final volume of 50ml each. Tubes were gently rolled to ensure thorough mixing and hence anticoagulation. Samples were centrifuged at 2000xg for 20 minutes to obtain plasma which was pooled and incubated at 37^{o}C for 30 minutes prior to preparation of fibrin monomer and kept at 37^{o}C throughout the procedure.

The Vivostat™ cartridge used in the automated device was carefully opened to release the biotinylated batroxobin powder, which was emptied into a glass scintillation vial and reconstituted with 0.3ml de-ionised water (equivalent to approximately 100BU/ml). 0.25ml of biotin-batroxobin solution (i.e. 25BU) was added to the 50ml aliquot of plasma and mixed at 37^{o}C for 5 minutes. The sample was then centrifuged at 3500 rpm for 5 minutes for formation of the fibrin monomer pellet which was subsequently harvested using a wooden swab stick by quickly stirring the sample and gradually progressing down the tube. The monomer was then transferred to a new vial, with any residual plasma removed by squeezing it against the side of the vial. 3.0ml of 0.2M sodium acetate buffer at pH 4.0 containing 24mM calcium chloride was then added to dissolve the fibrin monomer by vortex mixing for approximately 2 to 3 minutes. The yield of final fibrin monomer volume was approximately 4 to 5ml.

The avidin agarose compartment of the Vivostat™ cartridge was emptied directly into

the vial containing the fibrin monomer solution. The fibrin monomer was mixed at 37°C for 5 minutes. The sample was then filtered through a 20µm pore filter to remove the avidin-agarose:biotin-batroxobin complex and the fibrin monomer filtrate was collected and was then ready for use.

2.8.1.3 Application

The applicator device allows co-delivery of the fibrin I solution with 0.75M carbonate/bicarbonate buffer (pH10) in a ratio of 7:1 which is optimal for polymerisation of the fibrin monomer. The neutral pH achieved and the presence of calcium ions causes the conversion of endogenous prothrombin to thrombin and the activation of endogenous factor XIII within the fibrin I solution. This polymerises fibrin I to fibrin II and enables covalent cross linking of the resultant sealant to occur within 3 minutes (Figure 1.13).

Several applicator devices have been developed for the delivery of novel fibrin sealant, some of which have been utilised in these studies. Prevention of PSAs with novel fibrin sealant rabbit and pig studies used the Vivostat™ applicator 100 series (100µl) set at the mid density spray (26µl/s). Whereas application comparison and fibrin sealant comparison studies used the Vivostat™ 300µl applicator. Further application modifications investigated in the application study are described in Chapter 6.

All study products were supplied by ConvaTec Ltd (A Bristol-Myers Squibb Company). Fibrin I concentrations of the Vivostat™ system derived fibrin sealants were determined by a turbidimetric assay by ConvaTec Ltd. This assay is a spectrophotometric method based on the selective precipitation of fibrin I using ammonium sulphate resulting in a suspension which can be measured turbidimetrically.

2.8.2 Tissucol®

Tissucol® (or Tisseel®, Immuno AG, Vienna, Austria) is a commercially available pooled-source two component fibrinogen glue consisting of human fibrinogen, bovine thrombin and aprotinin, and calcium chloride. This study investigated the use of Tissucol

® using two bovine thrombin concentrations, 4 and 500 units.

Tissucol® was reconstituted with a bovine aprotinin solution at 37°C whilst thrombin was reconstituted in a calcium chloride solution at 37°C. Delivery was achieved via a Duploject system (Figure 1.12 Introduction), with mixing of the Tissucol® and thrombin solutions within the application system, resulting in the release of a fibrinogen glue.

2.8.3 CRYO

"CRYO" is the name given to fibrin sealants which utilise cryoprecipitation to obtain fibrinogen from donor blood rather than enzymatic methods. Traditionally they are autologous, in that they are prepared from a single blood donor, and hence avoid viral contamination problems. As with Tissucol®, exogenous bovine thrombin is required for fibrin I production, with 4 and 500 units thrombin used in the studies reported in this thesis.

2.9 Tissue Macrotomy

Once the resected experimental tissues had been fixed the total length of each experimental site was recorded, with one experimental site equal to abraded peritoneal wall together with apposed abraded uterine horn (rabbit studies) or apposed abraded colon, caecum or stomach (pig studies). Starting at an arbitrary point outside of the experimental site, the complete experimental site was cut transversely into slices of uniform thickness using a specially designed macrotome (Figures 2.15 and 2.16). Each slice was placed, with the same cut surface down, in a histology cassette and sequentially numbered.

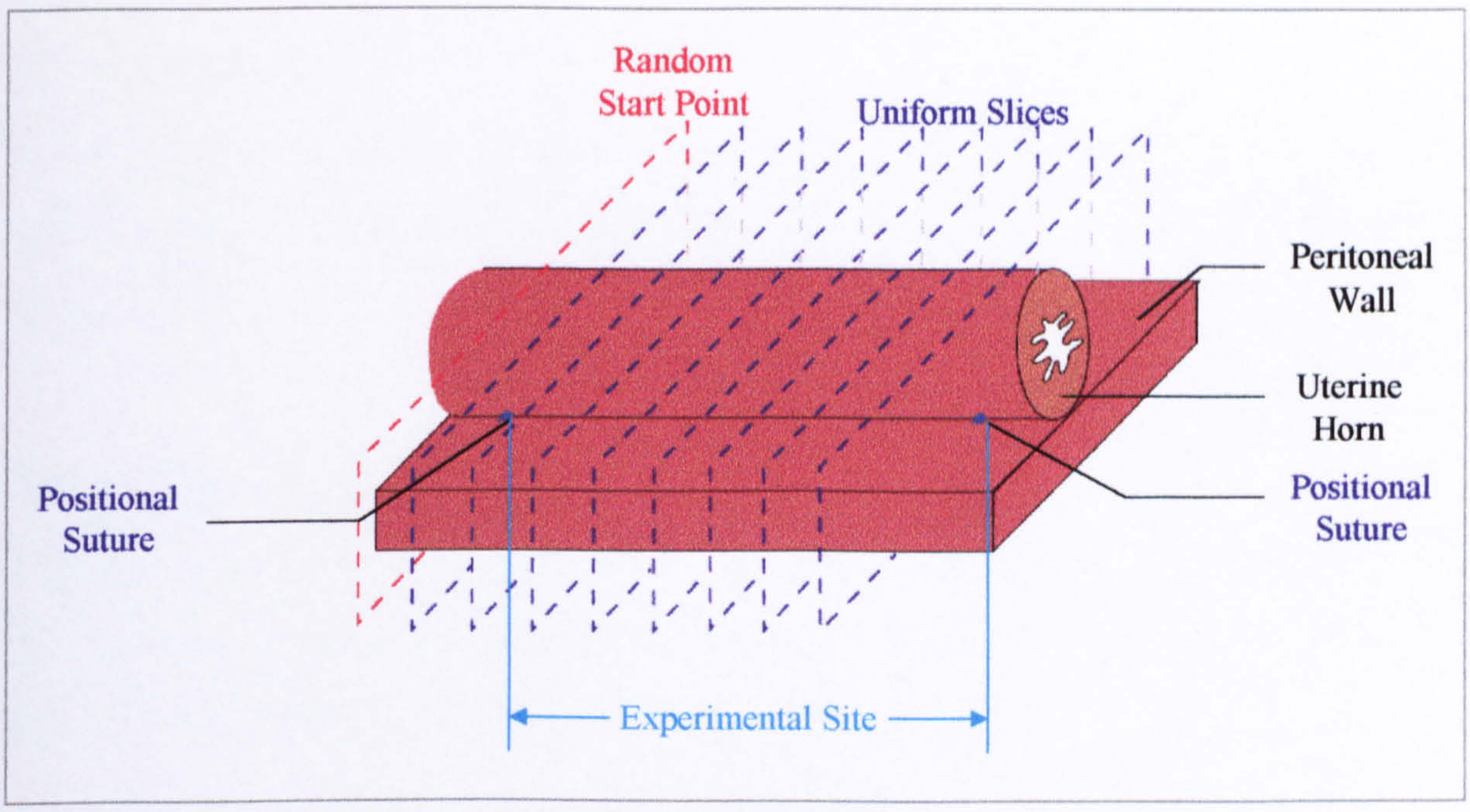


Figure 2.15: Uniform random slicing of the experimental site.

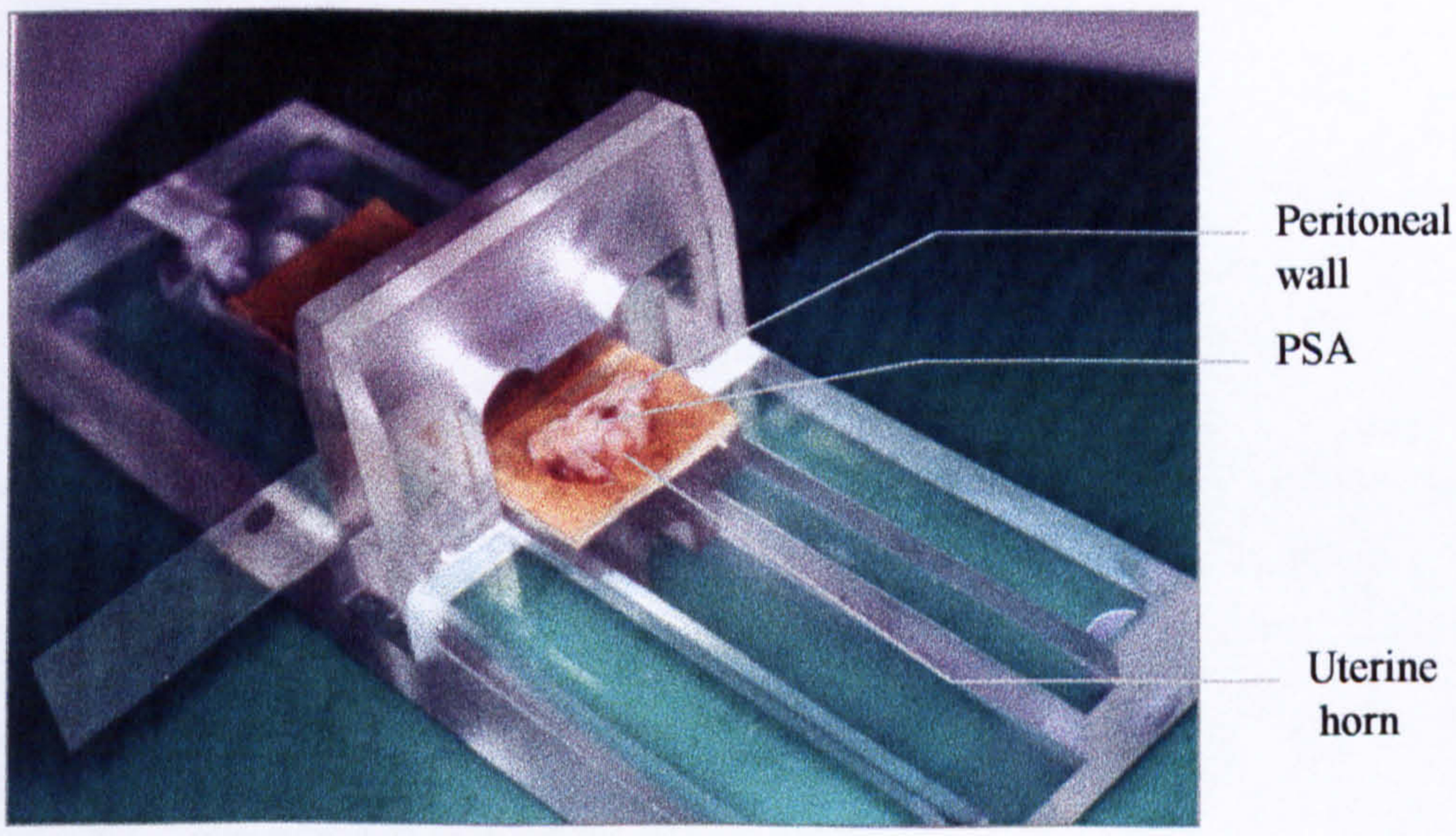


Figure 2.16: Macrotome and macroscopic transverse tissue sections.

2.10 Histology - Paraffin Wax

Dependent on the study, either odd numbered tissue slices (Pathogenesis of PSAs and Prevention of PSAs with novel fibrin sealant studies) or all slices (all other studies) were processed to paraffin wax for microscopical descriptive and Cavalieri's analysis. Paraffin wax is used routinely for histological processing due the large number of tissue blocks which can be processed in a relatively short time, under minimum supervision, ease of sectioning and the comparatively cheap cost of paraffin wax (Bancroft and Stevens, 1982).

2.10.1 Processing

All tissues were processed to paraffin wax using a Tissue Tek® VIP 2000 Tissue Processor (Bayer Diagnostic, Basingstoke, UK) according to standard procedures (Appendix II).

2.10.2 Embedding

Processed tissues were embedded using a wax embedding centre (Shandon, Runcorn, UK). Care was taken during embedding to ensure that the orientation of the transverse tissue slices was maintained.

2.10.3 Microtomy

5μm sections were cut from each block using a rotary microtome (Reichert-Jung 2030), with each section consisting of a full transverse section of tissue.

2.10.4 H&E Staining

Sections for Cavalieri's analysis of tissue zone volumes were stained with haematoxylin and eosin using Shandon Varistain 24-4 (Shandon, Runcorn, UK) according to standard procedures (Appendix II).

2.10.5 Mallory's Phosphotungstic Acid Haematoxylin (PTAH) Staining of Wax Sections for Fibrin Detection

Mallory's PTAH is routinely used for the detection of fibrin in wax sections. It is essentially made up of three components: haematoxylin, phosphotungstic acid and water, with the acid acting as the mordant to oxidise the haematoxylin to haematein. The PTAH staining method used in this study is a slightly modified version of Mallory's original method.

Procedure

Wax sections from pathogenesis of PSAs and prevention with novel fibrin sealant studies were stained with PTAH for fibrin detection.

Slides were de-waxed in xylene, rehydrated in decreasing grades of industrial methylated spirit (IMS) and taken to tap water following standard procedures. Slides were sequentially placed in 0.5% potassium permanganate for 5 minutes, 2.5% Iron Alum for 20 minutes and 1% Oxalic Acid for 3 minutes. Slides were then washed in distilled water before being placed in filtered naturally oxidised PTAH at room temperature overnight or at 60°C for 2 hours. All slides were then blotted using fibre-free papers, quickly dehydrated in increasing grades of IMS, cleared in xylene and mounted.

Fibrin was stained blue and collagen orange red. In addition, muscle stained blue, elastin and reticulin orange brown, basement membranes orange, osteoid orange red and cartilage variable shades of red.

2.10.6 Van Gieson Staining of Wax Sections for Collagen Detection

Van Gieson's stain is the simplest method for the detection of collagen in histological preparations. However it has the disadvantages of staining young collagen light pink instead of deep red as it does mature collagen and the tendency of the stain to fade, irrespective of the mounting medium used (Drury and Wallington, 1980).

Procedure

Wax sections from pathogenesis of PSAs and prevention with novel fibrin sealant studies were stained with Van Gieson for collagen detection.

Slides were de-waxed in xylene, rehydrated in decreasing grades of IMS and washed in tap water following standard histological procedures. Slides were stained in Celestine Blue solution for 5 minutes, then rinsed in distilled water. They were then placed in Cole's Haematoxylin for 2 minutes, washed in tap water and then differentiated in acid

alcohol for about 2 minutes. This was followed by washing slides well in tap water and then staining with Van Gieson solution for 6 minutes. Slides were then blotted using fibre-free papers and very quickly dehydrated through increasing grades of IMS, cleared in xylene and mounted.

Results obtained showed collagen stained red and fibrin yellow. Additionally muscle, elastin, reticulin, cartilage and basement membranes stained yellow and osteoid red.

2.11 Immunohistochemistry

Immunohistochemistry has found a niche in cases were routine and special histological stains fail to provide the specificity required for diagnosis. This technique utilises antibodies for the detection of structures of interest. Hence immunohistochemistry provides a highly specific and specialised identification system, which can be visualised using standard light microscopy.

Two antibodies are employed:

i. primary antibody directed to detect the structures of interest (antigen); and
ii. secondary antibody which is directed against the primary antibody and has an enzymatic component, which, when a substrate is added, acts a tracer.

Several enzymes have been used as markers, however the most frequently used, and the one used in these studies, is horseradish peroxides (Figure 2.17).

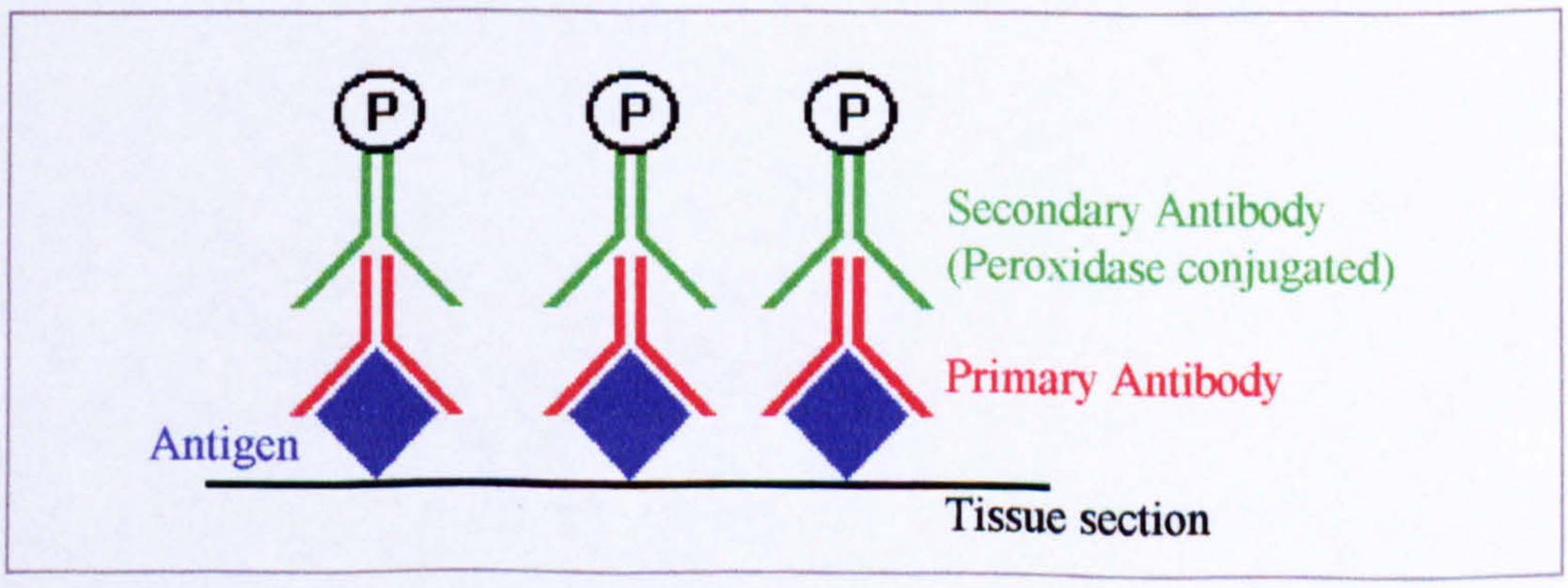


Figure 2.17: Immunohistochemistry principle.

A limiting factor for the use of immunohistochemistry is the type of tissue fixation used.

Routinely histological specimens are fixed using neutral buffered formal saline, however this fixation technique introduces problems with regard to immunohistochemical detection. Although the actual molecular mechanisms occurring during tissue fixation by formaldehyde is not completely understood, it is generally agreed that there is a loss of antigenicity, primarily due to the masking of antigens present. In order to overcome this problem several antigen retrieval techniques have been developed which can be grouped as either heat treatment (microwave and pressure cooker) or enzymatic treatments which are generally proteolytic.

In pathogenesis of PSAs and prevention with novel fibrin sealant studies, formalin fixed, paraffin wax embedded sections were analysed for fibrin and collagen using immunohistochemical methods. Antigen retrieval techniques were used for both detection systems, with proteolytic treatment used for fibrin detection and pressure cooker treatment used for collagen detection, as recommended by suppliers of the primary antibodies.

Primary antibodies used were obtained from commercial sources (refer to sections 2.11.1 and 2.11.2) and, as no antibodies directed against rabbits were available, anti-human antibodies were used. In order to validate these antibodies for the detection of rabbit fibrin and collagen, rabbit and human control tissues were used.

2.11.1 Immunohistochemical Detection of Fibrin

For the detection of fibrin in formalin fixed, paraffin wax embedded rabbit tissues an anti-human fibrin IgG1 (American Diagnostica Inc, Greenwich, USA. Supplied by Alpha Laboratories Ltd, Eastleigh, Hampshire, England) was assessed. This has been shown to detect fibrin in snap frozen human cardiac allografts (Faulk *et al.*, 1992) and was the most appropriate fibrin specific monoclonal antibody available at the time of investigation with the potential to detect fibrin in formalin fixed, paraffin embedded tissues.

Procedure

Sections were dewaxed in xylene, rehydrated through decreasing grades of IMS and taken to 37°C distilled water. Slides were transferred to 0.1% protease (Type XXIV) solution (Sigma-Aldrich Company Ltd, Poole, England) at 37°C for 5 to 10 minutes and then washed well in running tap water to stop protease activity.

Slides were subsequently washed in phosphate buffered saline (PBS) (pH 7.3) for 10 minutes. Endogenous peroxidase activity was blocked by incubating sections in freshly made 0.3% hydrogen peroxide in methanol for 20 minutes. Slides were then washed twice in distilled water for 5 minutes and twice in PBS for 5 minutes.

To reduce non-specific background staining, sections were covered with normal horse serum (Vector Laboratories, Peterborough, England) (15µl normal horse serum in 1ml PBS) for 20 minutes, then excess serum was removed by blotting with fibre-free paper.

Slides were incubated with diluted monoclonal anti-human fibrin IgG1 for 1 to 2 hours at room temperature. Slides were then washed twice in PBS for 5 minutes. This was followed by incubation with peroxidase labelled secondary antibody (Vector Laboratories, Peterborough, England) (15µl normal horse serum in 1ml PBS plus 5µl biotinylated anti-mouse serum) for 30 minutes at room temperature.

During the incubation period the Elite ABC (Vector Laboratories, Peterborough, England) was prepared. After the incubation period slides were twice washed in PBS for 5 minutes and then incubated for 30 minutes with Elite ABC at room temperature.

Slides were again washed twice in PBS for 5 minutes and then developed in 3,3 Diaminobenzidine (DAB) (Vector Laboratories, Peterborough, England) for 5 minutes at room temperature. Slides were then washed twice in water for 5 minutes and counter stained with Cole's haematoxylin for 1 minute. Finally slides were washed in running tap water for 2 minutes, dehydrated through increasing grades of IMS, cleared with xylene and mounted.

2.11.2 Immunohistochemical Detection of Collagen

For the detection of collagen in formalin fixed, paraffin wax embedded rabbit PSA tissues a human (interstitial) collagen I, II, III clone MCI-HA monoclonal antibody (Monosan, Netherlands, supplied by: Bradshaw Biologicals Ltd, Loughborough, England) was assessed. This antibody was chosen primarily due to its ability to detect all interstitial collagens and its potential to detect collagen in formalin fixed, paraffin embedded tissues.

Procedure

Slides were dewaxed in xylene, rehydrated in decreasing grades of IMS and taken to distilled water following standard procedures. 1600ml of 0.01M sodium citrate buffer (pH 6.0) was brought to the boil in a pressure cooker, slides were placed into a metal staining rack and lowered into the pressure cooker, ensuring that slides were well immersed in citrate buffer. The lid was then locked and slides were heated for 1 minute once pressure had been achieved. The pressure cooker was then removed from the heat source, run under cold water until the pressure had dissipated. Slides were then removed and placed immediately into distilled water.

Slides were washed in PBS for 5 minutes. Endogenous peroxidase activity was blocked by incubating slides with freshly made 1.5% hydrogen peroxide in methanol for 10 minutes. Slides were then washed twice in distilled water and subsequently PBS for 5 minutes.

To reduce non-specific background staining slides were covered with normal horse serum (refer to section 2.11.1.1) for 20 minutes, with excess serum then blotted from the slides using fibre-free paper.

Slides were incubated with a human (Interstitial) collagen I, II, III Clone MCI-HA monoclonal antibody for 1 to 2 hours at room temperature. Slides were then washed

twice in PBS for 5 minutes, followed by incubation with peroxidase labelled secondary antibody (refer to section 2.11.1.1) for 30 minutes at room temperature.

Elite ABC (refer to section 2.11.1.1) was prepared and, following incubation, slides were twice washed in PBS for 5 minutes and then incubated for 30 minutes with Elite ABC at room temperature. Slides were again twice washed with PBS for 5 minutes and then covered with DAB (refer to section 2.11.1.1) for 5 minutes at room temperature.

They were washed twice in water for 5 minutes each and counter stained with Cole's haematoxylin for 1 minute. Finally slides were washed for 2 minutes in running tap water, dehydrated through increasing grades of IMS, cleared in xylene and mounted.

2.12 Histology - Resin Processing

For pathogenesis of PSAs and prevention with novel fibrin sealant studies, quantitative analysis of several different cell types was performed using the stereological "optical brick" method for determining cell number density (refer to section 2.14.2). This required a relatively thick tissue section (25μm). Although paraffin wax is the routine embedding medium used in histological investigations, it needs to be removed during staining procedures. For thick sections (such as 25μm) removal of the embedding medium causes tissue collapse, hence for this analytical technique an embedding medium which remained during staining was required.

Many resins, including glycol methylacrylate, do not need to be removed for adequate staining, hence tissue ultrastructure is maintained. However care needs to be taken with regard to the type of resin selected as some processing fluids used to prepare tissue for resin embedding cause shrinkage. Also some resins are unsuitable for thick sections due to their brittle nature. Glycol methylacrylate, as used in these studies, optimises sectioning of thicknesses of 25μm or greater with minimum tissue processing (Mayhew & Gundersen, 1996)

2.12.1 Tissue Sampling

A finger of tissue was taken from the centre of even numbered experimental slices, comprising of the injury sites from both the peritoneal wall and uterine horn (Figure 2.18). For non-adhered tissues, injury sites on the peritoneal wall and uterine horn were aligned and a sample taken from the central portion of both.

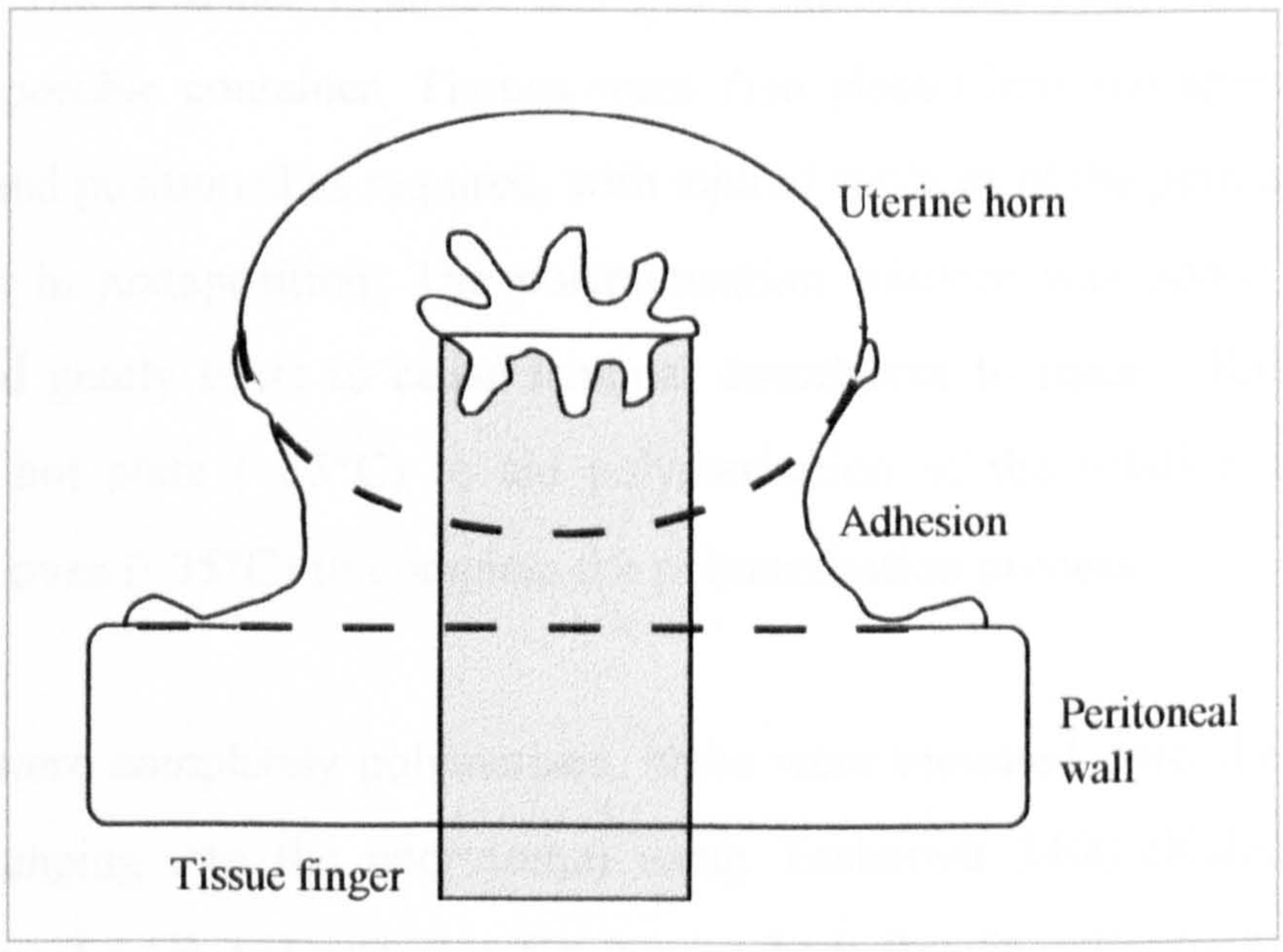


Figure 2.18: Schematic representation of tissue finger taken for resin processing.

Tissue fingers were placed into separate glass vials containing 10% neutral buffered formal saline labelled with the relevant animal number and tissue slice number.

2.12.2 Tissue Processing

Technovit 7100 (Kulzer Histo-Technik; supplied by TAAB Laboratories Equipment Ltd, Reading, England) is a cold-polymerising resin based on hydroxyethylmethacrylate. The main properties it possesses are even penetration and polymerisation through specimens, low temperature polymerisation (23-37°C), reproducible and reliable embedding and its suitability to remain present during routine staining procedures.

There are 4 main stages of processing tissues to resin:

i. Dehydration - via immersion in increasing grades of IMS 70%, 80% and 95% for approximately 12 hours each

ii. Pre-infiltration - achieved by mixing equal amounts of absolute IMS and base liquid Technovit 7100 and incubating tissues in the resulting solution at room temperature for approximately 12 hours

iii. Infiltration - immersion of tissues in infiltration solution, consisting of 1g (1 sachet) of hardener I dissolved in 100ml of base liquid Technovit 7100

iv. Polymerisation - 1ml hardener II added and gently stirred into 15ml of infiltration solution in a disposable container. Tissues were then placed into the appropriately labelled moulds and positioned as required, with injured surfaces of the peritoneal wall and uterine horn in juxtaposition. The polymerisation solution was added to each mould slowly and gently so as to cause minimal disturbance to tissues. Racks were transferred to a hot plate (~35°C) to aid polymerisation of the solution and then transferred to an oven (~35°C) to complete the polymerisation process.

Once resin blocks were completely polymerised, stubs were mounted onto the back of each block (for clamping into the microtome) using Technovit 3400 (Kulzer Histo-Technik; supplied by TAAB Laboratories Equipment Ltd, Reading, England). 5ml of Technovit 3040 liquid was added to 10g of Technovit 3040 powder and stirred quickly but gently. A layer of the Technovit 3040, approximately 2mm, was applied to the back surface of each resin block and a stub pressed into the Technovit (Figure 2.19). Completed blocks were then left to polymerise completely, after which they were labelled and blocks were then removed from the moulds.

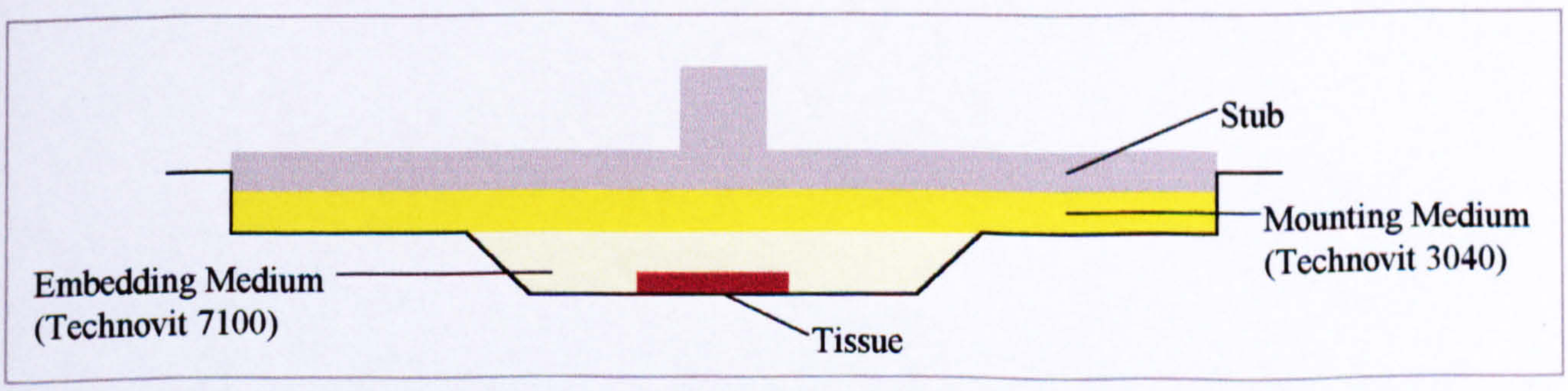


Figure 2.19: Embedding set-up for resin (Technovit 7100 and 3040).

2.12.3 Microtomy

A 25μm section, containing a complete transverse section of tissue was cut from each block using a Mod. 1140 Autocut microtome (Reichert-Jung) and mounted onto a slide. To aid sectioning, the surface of the resin block was slightly moistened with water. Slides were left on a hot plate at 70-80°C for approximately 2 hours and subsequently transferred to an 37°C incubator overnight to ensure complete adherence of sections to slides.

2.12.4 Giemsa Staining

Giemsa, a Romanowsky stain, differentially stains cellular granules and makes subtle distinctions in staining shading. It is comprised of a mixture of eosin and methylene blue solutions and it is the binding between the dyes and tissue components and between the dyes themselves which is responsible for the differential staining of cellular components. Used in collaboration with a weak acetic acid solution, further differentiation can be achieved, especially for the detection of eosinophil granules. The alcoholic properties of Giemsa also aid the penetration of the stain through the 25μm resin sections.

Procedure

A modified version of Giemsa's technique was used. Slides were placed directly into 0.005% Giemsa solution at 56°C for 40 minutes and then quickly rinsed twice in distilled water. Slides were then differentiated in a very weak acetic acid solution (1:10 000 acetic acid to distilled water) for 10 minutes, gently agitated throughout. They were then quickly rinsed twice in distilled water, blotted using fibre-free papers, thoroughly dehydrated by incubation at 37°C for 24 hours and mounted.

2.13 Tissue Zones

Microscopic analysis of transverse sections revealed several different, discernible tissue area components which could be analysed stereologically (Figure 2.20) These were defined and classified as follows:

i. PSA - any tissue, arising from the injury sites, which connected the two surfaces.
ii. Tissue generation - regenerative tissue growth attached either to injury site but not connecting the two.
iii. Damage zone - the area between the line of the original serosal surface and the limit of actual visual tissue damage.
iv. Reaction zone - the area between the limit of actual tissue damage and the limit of tissue changes not amounting to frank damage but identifiable as changes from normal (colour, cellular infiltration).
v. Peritoneal cell concentration zone - areas of abnormal cellular concentrations, mainly neutrophils, eosinophilic cells, and macrophages within the peritoneum.
vi. Uterine horn haemorrhage - haemorrhage located in the villus structures on the lumenal surface of the uterine horn.
vii. In treated cases, fibrin sealant remaining and fibrin sealant associated cell concentration/reaction was quantified.

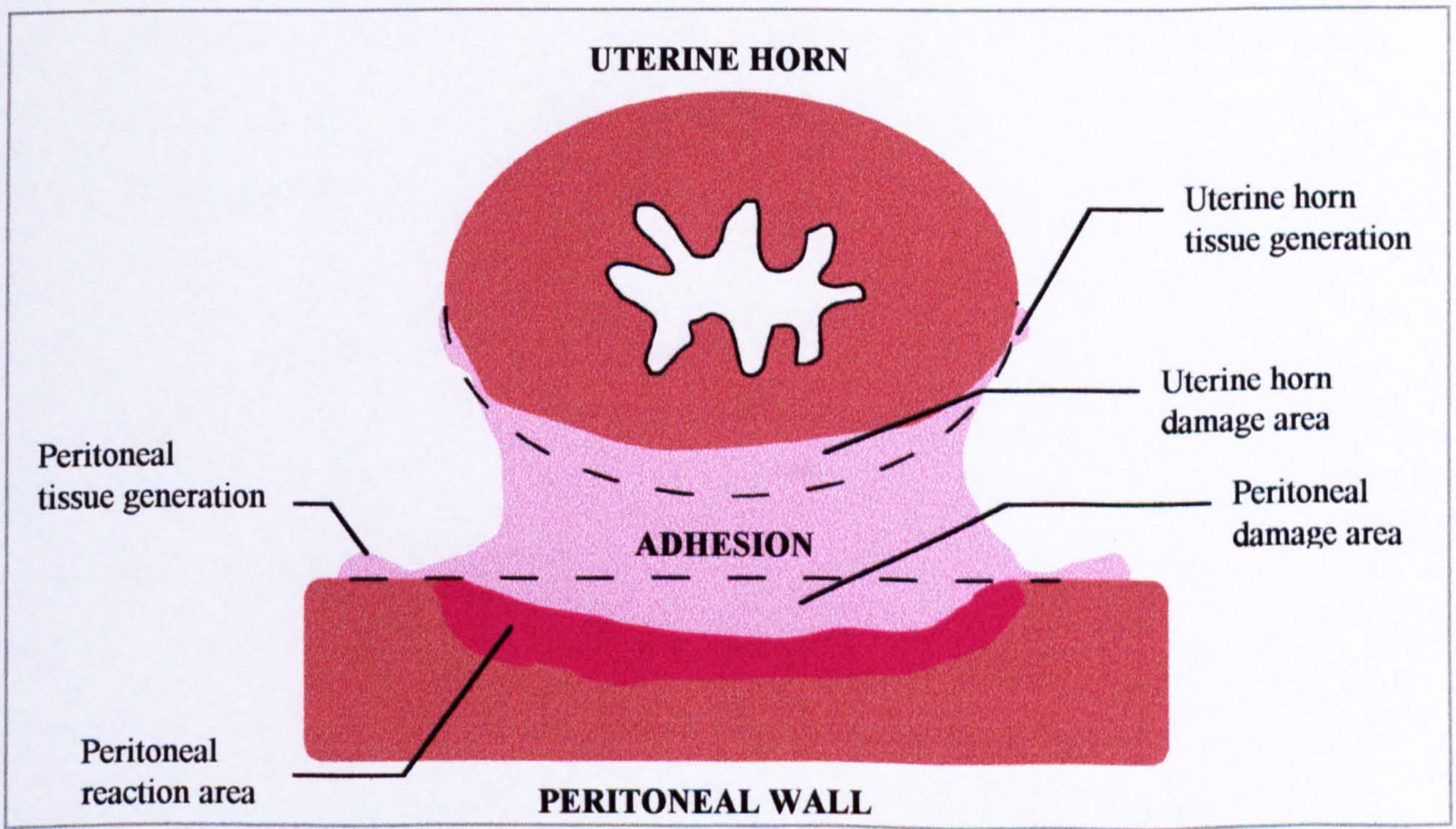


Figure 2.20: Schematic representation showing tissue zones in the rabbit uterine horn model. Corresponding zones could be distinguished in the experimental sites (peritoneal wall to colon/caecum and stomach) in the pig abrasion model.

2.14 Stereological Analysis

The benefits of quantitative compared to qualitative data are many, but in summary can be described as the difference between absolute and observational knowledge. This is especially important when comparing the effects of different treatments, for example, potential PSA preventative agents. Qualitative data has the potential to be biased and imprecise, hence interpretation of data can be difficult, particularly when comparing different potential PSA preventative agents assessed by different qualitative methods. These problems are eliminated by unbiased and precise quantitative analysis.

The field of stereology is concerned with obtaining quantitative 3 dimensional histological information derived from measurements made on the 2 dimensional planer surfaces of tissue sections (Gundersen *et al.*, 1988). In order for data generated to be accurate it needs to be:

i. **unbiased**, that is without systematic deviation from the true value. For example, two separate investigations are made to estimate the same quantity (Figure 2.21). Investigation X is biased and the value obtained is not the true value whereas investigation Y is unbiased with the true value found. The difference between value X and Y is the magnitude of bias, easily identifiable in this example but not during experimental practices.

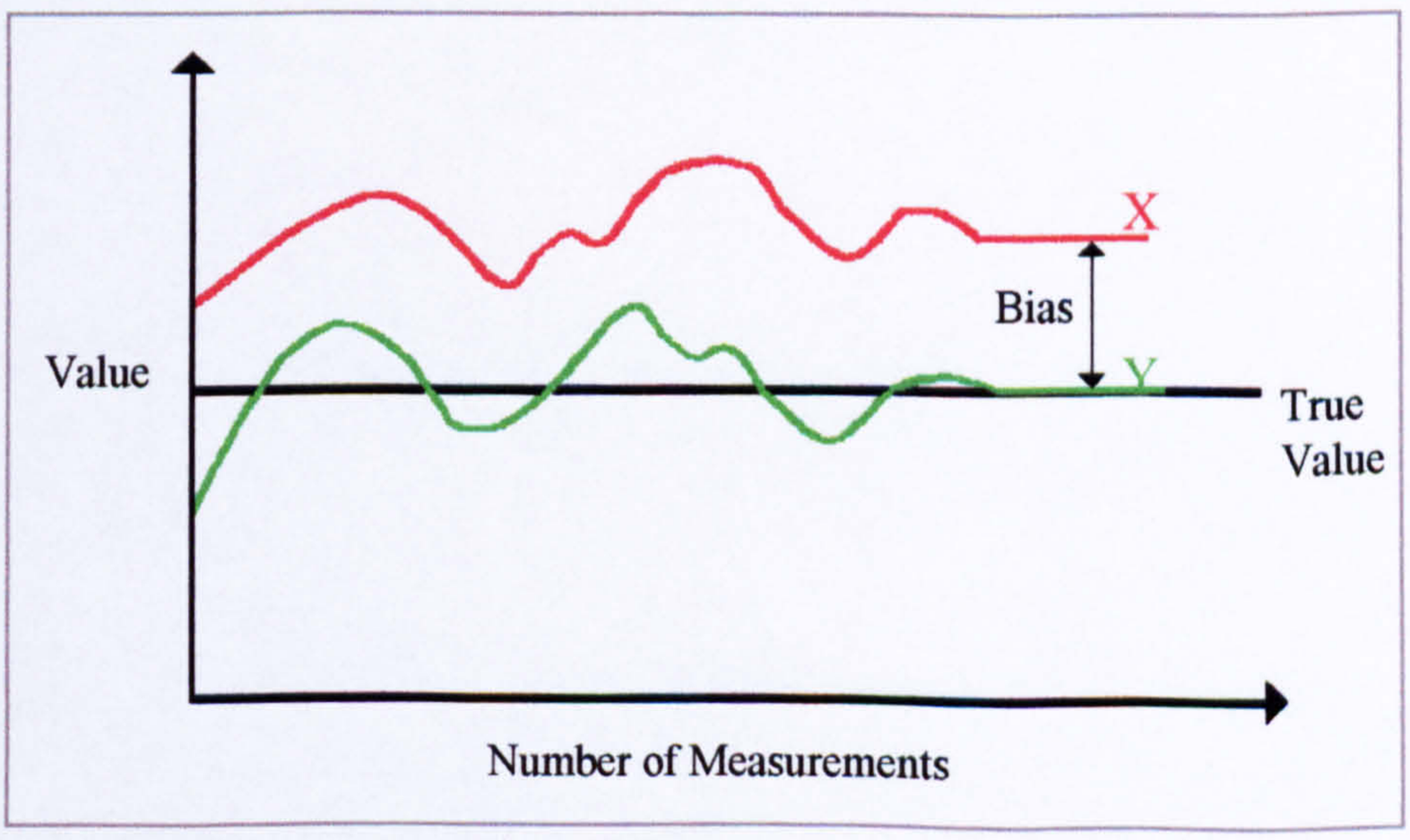


Figure 2.21: Effect of bias on quantitative analysis.
(Taken from Howard and Reed, 1998).

ii. **precise**, that is having low measurement variability, with precision decreasing the number of measurements and hence time required to obtain data.

Dual benefits of unbiased and precise estimates of structural quantities is supplied only by stereology and no other technique (Mayhew, 1991a). However for both of these requirements to be met a few specific demands need to be fulfilled (Gundersen *et al.* 1988) during analysis. If these are not taken into account, inaccurate results will be obtained, the identification of which may be difficult. The very worse case of this is precision combined with bias as results are highly inaccurate but appear to be very good (Figure 2.22).

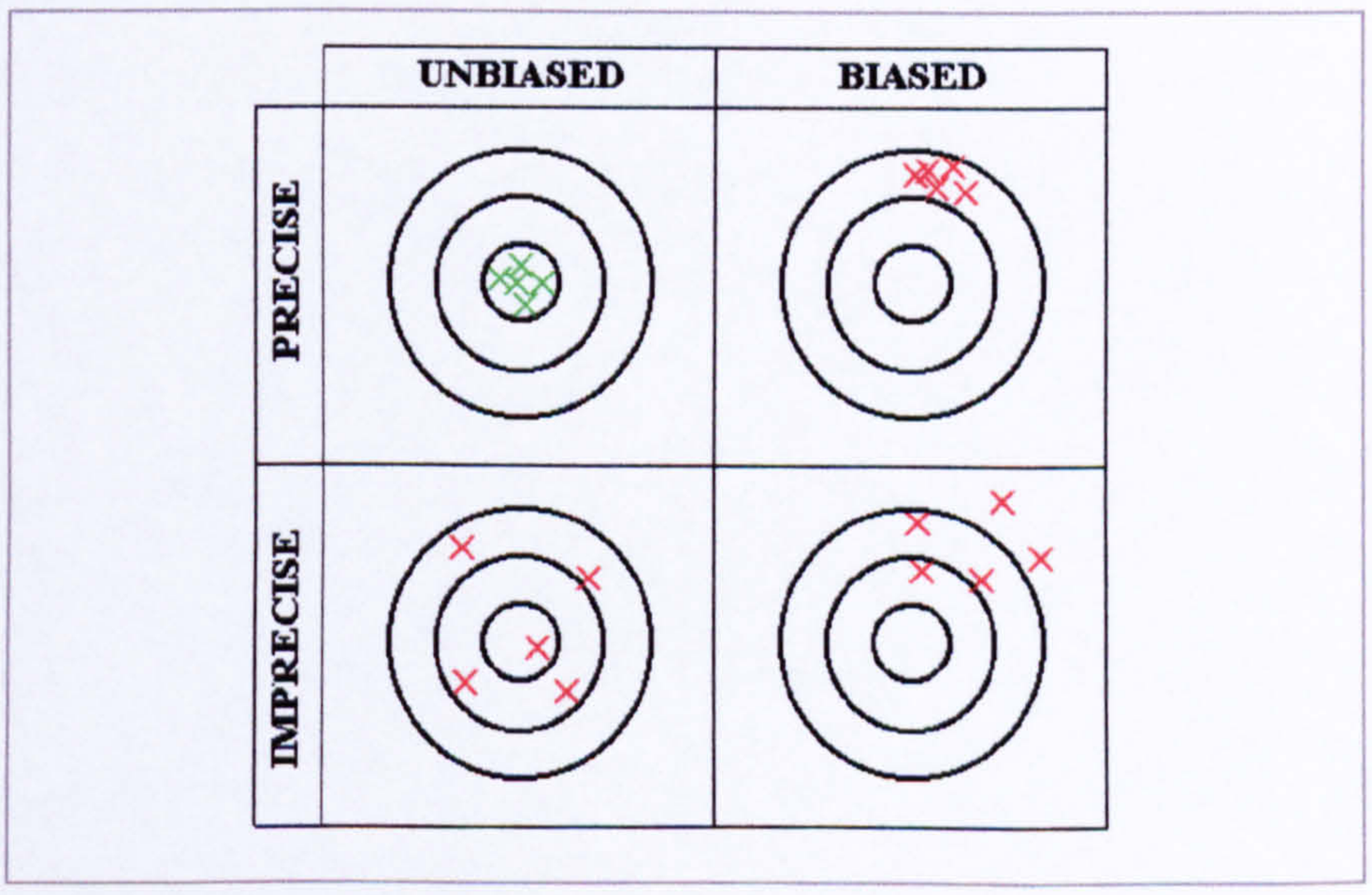


Figure 2.22: Importance of unbiased and precise measurements for obtaining accurate data. (Modified from Howard and Reed, 1998).

For precise and unbiased estimates to be calculated several criteria need to be met with regard to the sampling technique utilised:

i. no assumptions should be made about geometry, orientation, size or spatial distribution of the objects to be measured. The only way to achieve this is to use a design based approach (Howard 1990).

ii. random sampling must be employed, that is, "to present all parts of the specimen an equal chance of being selected for examination" (Stuart, 1976). It is impractical, when obtaining microscopic quantitative data, to analyse the whole object of interest, for example, to count all cells present within a PSA when the total number could be

millions of cells per millimetre of tissue. To circumvent this impracticality, discrete samples are taken from the object of interest in a uniform random manner. Using uniform random sampling decreases variance after analysis of a few samples and hence increases the precision of the estimate compared to a random random sampling technique.

Stereological techniques utilised in these studies were Cavalieri's Principle for determination of absolute volumes and the Optical Disector for numerical density, both of which are described below.

2.14.1 Cavalieri's Principle

The determination of the volume of an object can be easily achieved for most objects via measurements of height and specific gravity. However for the calculation of volumes within volumes, for example, calculation of the damage zone within peritoneal tissue, these techniques are redundant. A method was developed by an Italian mathematician, Cavalieri (1598 to 1647) (Gundersen *et al.*, 1988) for simple and accurate calculation of absolute volume and permits the determination of volumes within volumes. Cavalieri showed that "the volume of an object may be estimated from parallel sections, a known distance apart, by summing up the areas of all cross sections and multiplying this by the distance apart of each slice".

Practically this technique involves the random application of a grid of uniformly placed points over the surface of each slice from a uniform randomly sliced object and counting the number of points associated with slice surface. This information together with the area associated with each point and mean thickness of each slices is used to calculate the absolute volume of the object.

Experimental tissues were uniformly random sliced throughout the length of each experimental site, with the mean slice thickness known (refer to sections 2.9 and 2.10 for rules to be observed). Previous studies have shown that efficient estimates may be

obtained even if as few as 5 or 6 slices per object are taken (Gundersen and Jensen, 1987). All cases in the studies reported in this thesis were therefore sliced to produce at least this number of slices. From each slice a section was produced and was viewed at a known magnification, using a modified microfiche reader (Canon FR 100). A grid of uniformly and constantly spaced points was placed, at random, over the image of each section (Figure 2.23).

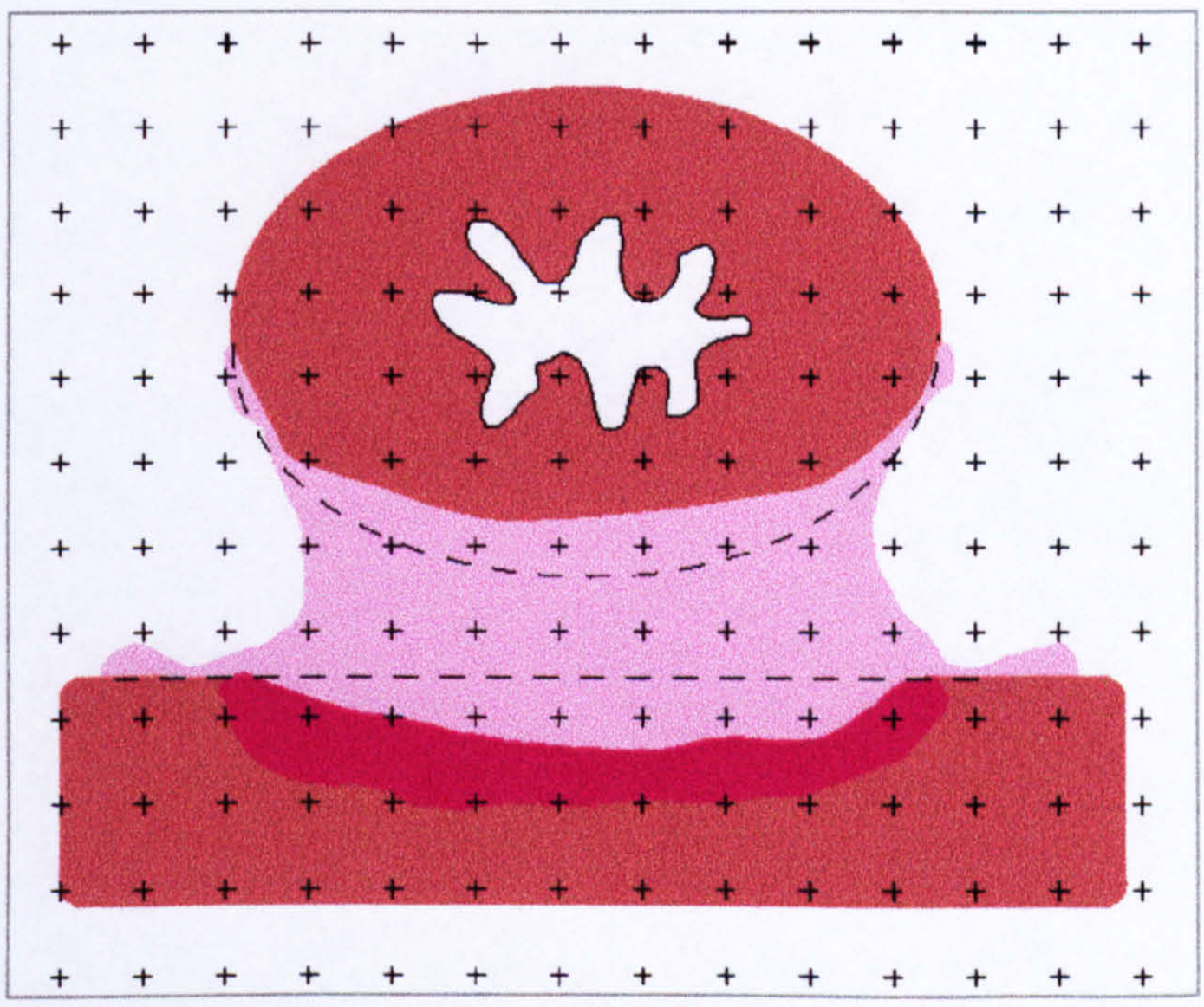


Figure 2.23: Schematic representation of uniform grid of points randomly applied over a transverse section.

The number of points (P) lying within the boundary of the area of interest was counted for all sections of the experimental site. These were totalled to obtain the entire number of points associated with this area of interest (ΣP). This value together with the area associated with each test point (aP) and mean thickness of each slice ($\bar{t}$) was used to calculate the reference volume for each area of interest (Vref):

$$V_{ref} = \sum P_{ref} \,.a(P_{ref}).\bar{t}$$

2.14.2 Numerical Density

For pathogenesis of PSAs and prevention with novel fibrin sealant studies numerical densities of basophilic cells, eosinophilic cells, macrophages, lymphocytes and fibroblasts within PSA and tissue generation zones for each experimental site were determined using the stereological method of the optical brick (Howard *et al.*, 1985).

Until the 1980's counting of particles in 3 dimensional space had been model based and required assumptions to be made about particle size, shape and spatial orientation (Mayhew, 1991a). Such assumptions resulted in many number estimates being inaccurate and biased. For example, when sectioning tissue for histological analysis, larger particles have a greater chance of being transected compared to smaller particles, in addition smaller particles have more chance of being totally included within a section thickness and therefore not displaying a profile on the section surface. Cigar shaped particles have greater probabilities of being transected transversely or obliquely rather than longitudinally and a perimeter of irregular particles can be transected by more than one plane creating potential difficulty in deciding whether or not individual particles appearing on that plane belong to the same parent particle (Mayhew, 1991a).

In 1980 Cruz-Orive proposed the use of a volume probe, which consisted of a stack of serial sections cut from a random starting point to count particles in 3-D. This system was independent of particle shape, size and orientation and hence eliminated the problems of previously used methods. This principle was further developed by Sterio (1984) who stated that "arbitrary particles of any size and shape are sampled with a uniform chance only with a 3 dimensional probe". He described the 3-D probe as a disector, that is, an unbiased counting frame (UCF) and a pair of serial sections a known distance apart. This design based disector principle proposed by Sterio (1984) was a major contribution to precise and unbiased counting of particles within a volume. However the efficiency of counting particles by the taking of physically separated serial sections can be increased by the use of a single thick section and optically sectioning through that section (Howard and Reed, 1998).

The first practical adaptation of this counting technique was demonstrated by Howard *et al.*, (1985) who introduced the "optical brick". The basic principle is the 3-D probe, of a known volume, samples the tissue of interest in a random uniform manner. The optical brick has three exclusion planes and three acceptance planes which are used for counting the number of profiles (cell nuclei in this study) present within its volume (Fig 2.24). The numerical density can then be calculated from measurements therefrom.

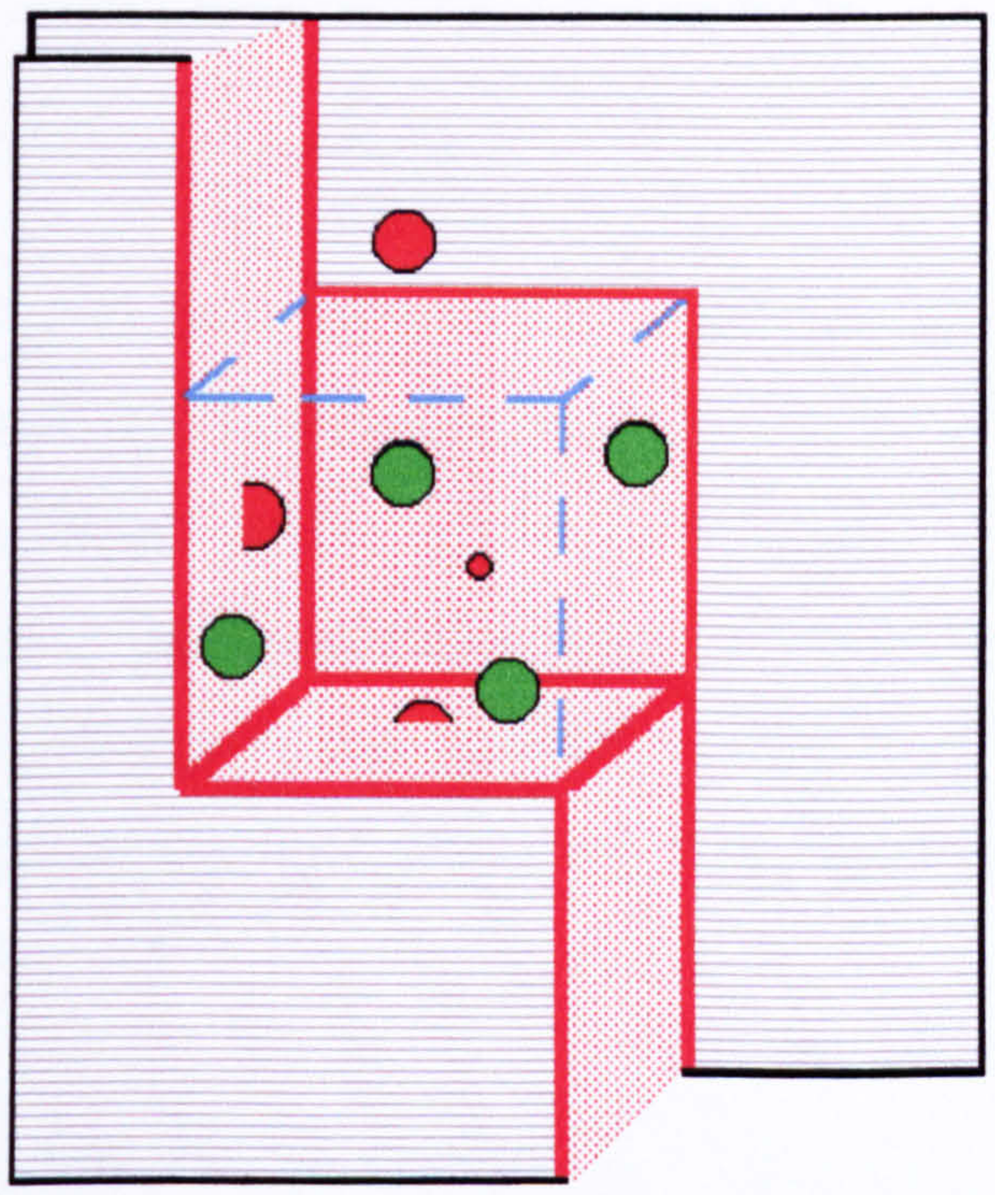


Figure 2.24: Optical brick.
(Adapted from Ansari, 1997).
The green spheres are those which would be counted since they do not intersect the exclusion planes (red) whereas the red spheres would not be counted since they intersect the exclusion planes.

Practically the "optical brick" used in these studies consisted of an UCF mounted on a monitor screen used in conjunction with a known reference depth of 15μm. The unbiased counting frame consists of two exclusion and two guard lines used for the unbiased counting of profiles (Figure 2.25), with a counting rule which states "profiles of features which intersect the frame but do not intersect the exclusion edge (solid line) are sampled. Features intersecting the image boundary will be rejected, since they would inevitably bias the estimate" (Gundersen, 1977).

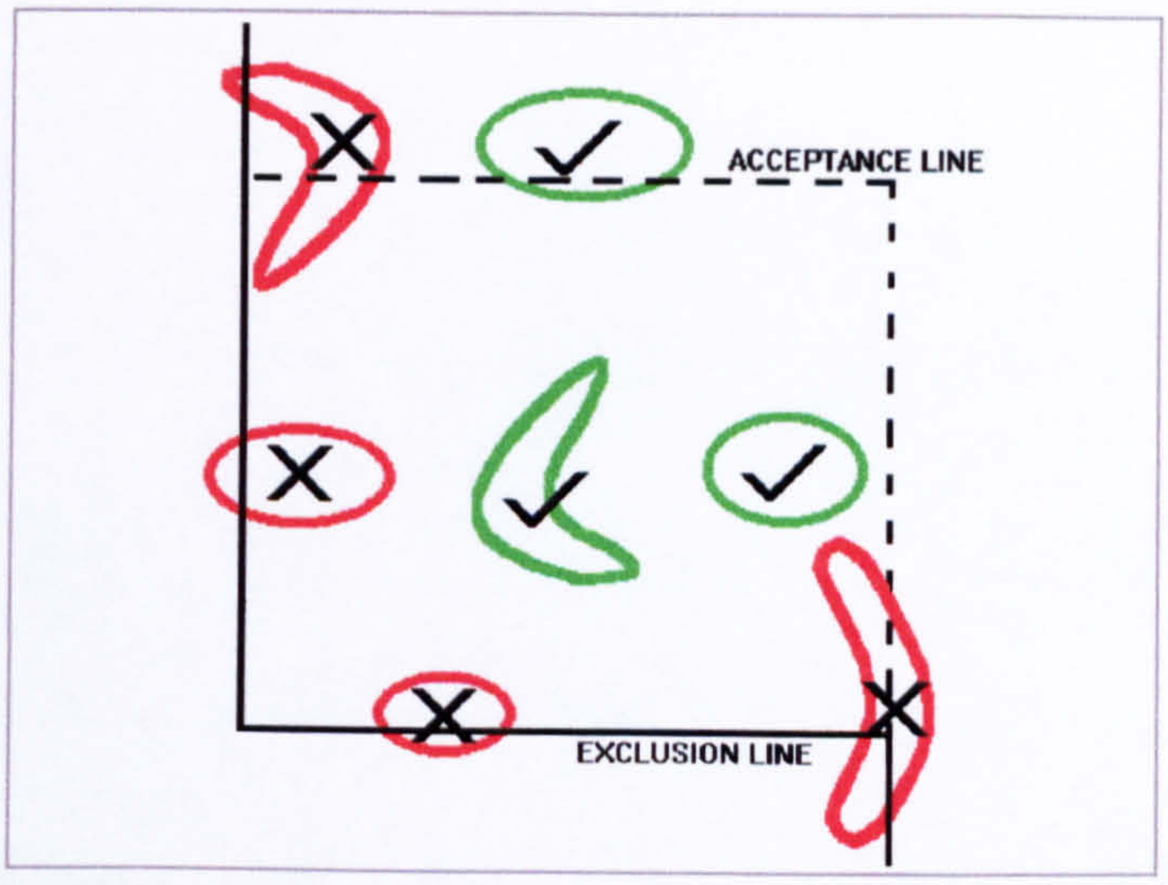


Figure 2.25: Unbiased counting frame (UCF).

Resin sections were viewed under oil immersion (x100 objective lens; NA 1.25), with colour images projected via a camera (Sanyo Colour CCD video camera, Model No: VCC 3900) onto a colour monitor (JVC, Model No: AV-20GB), with a final magnification of x1250. Initially the tissue zone to be analysed was located at low magnification and a starting point was randomly chosen outside of this zone (Figure 2.26). It this way analysis commenced from a truly random starting point within the area of interest.

Once the first sample point was found, the section was focused through until the first nucleus of a cell of interest (either at the top or bottom of the section) came into maximum focus. The microcator (Sylvac, Sylvac System), an instrument used for the measuring of stage movement, hence the reference depth, was then set to zero and counting commenced from this point. The section was focused through until the nucleus of a cell of interest came into maximum focus and that cell recorded provided it was within the reference depth of 15μm and conformed to UCF counting rules. This procedure was repeated until the whole of the reference depth was covered.

Once that sample point was completed, the section was moved in a uniform manner by tessellating across the whole of the tissue, repeating the counting procedure (Figure 2.26).

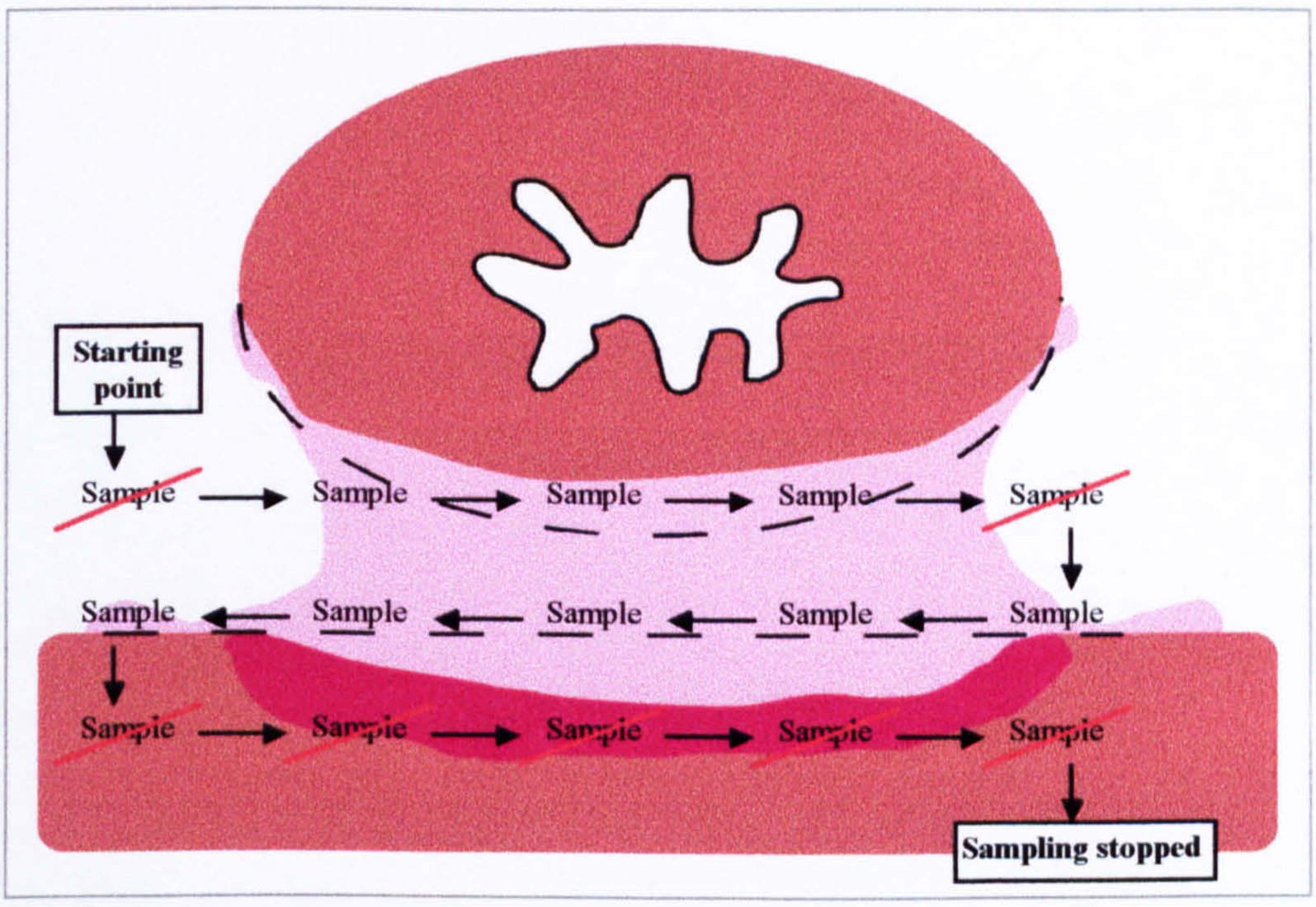


Figure 2.26: Uniform random sampling of PSA for numerical density estimation.

Numerical densities were calculated as follows.

i. Calculation of the optical brick volume used:

$$Volume\ of\ Optical\ Brick = Area\ of\ UCF \cdot Reference\ Height$$
$$V_f = A_f \cdot h$$

ii. Calculation of the volume of tissue sampled:

$$Volume\ of\ Tissue\ Sampled = Volume\ of\ Optical\ Brick \cdot Number\ of\ UCFs\ Counted$$
$$V_{Tiss} = V_f \cdot N_f$$

iii. Numerical density is then derived using:

$$Numerical\ Density = \frac{Number\ of\ Profiles\ Counted}{Volume\ of\ Tissue\ Sampled}$$
$$N_V = \frac{Q^-}{V_{Tiss}}$$

Total number can then be determined using the numerical density and absolute volume determined using Cavalieri's principle.

$$Total\ Number = Numerical\ Density \cdot Reference\ Volume$$

$$N_{TOT} = N_V \cdot V_{REF}$$

2.14.3 Length Density

For studies investigating PSA development and prevention with novel fibrin sealant length density and subsequently total length of vessels (blood and lymphatic) within PSAs and tissue generation were calculated using stereological methods.

The theory behind this methodology is that a 2-D probe, for example a histological section, hits an object in proportion to its length (Figure 2.27). Thus the number of intersects of the object per unit area of the section is related to the length of the object within the reference space. However section orientation can cause problems, with intersect number varying with differing section orientation. To avoid this problem and to ensure unbiased length estimation sections should be of uniform random orientation, which practically is the usual case (Howard, 1998).

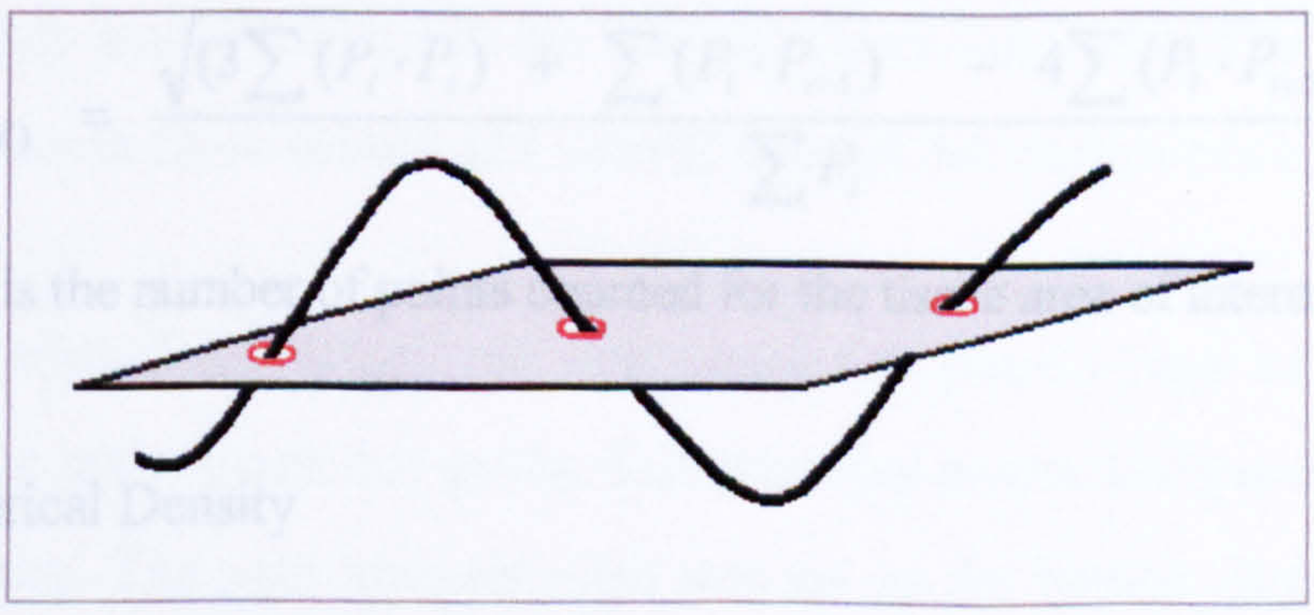


Figure 2.27: Intersection of a linear feature by a random plane/section (Taken from Howard, 1998).

To estimate length density tissues are uniformly randomly sampled using UCFs. The number of points within UCFs are counted and used to calculate length density as follows:

$$Length\ Density = 2 \cdot \frac{Number of\ Points}{UCF\ Area}$$

Due to time limitations only preliminary analyses were performed. Two left hand side experimental sites were selected from each sample time point with 3 slides (L1, L5 and L9) analysed only. Approximately 15 samples were taken for each case with tissues sampled in an uniform random manner. Length density estimations and calculations were performed using a purpose designed digital stereology package (Kinetic Imaging, Liverpool, UK). Total length was subsequently calculated by multiplying length density by total volume (calculated by Cavalieri's method).

$$Total\ Length = Length\ Density \cdot Reference\ Volume$$

2.14.4 Coefficient of Error

To determine the precision of the quantitative counting methods used, the coefficient of error (CE) (Gundersen and Jensen, 1987; Matheron, 1965) was calculated for each technique using the following equations:

i. CE for Volume

$$CE_{(Vref)} = \frac{\sqrt{(3\sum(P_i \cdot P_i) + \sum(P_i \cdot P_{i+1}) - 4\sum(P_i \cdot P_{i=2}))/12}}{\sum P_i}$$

where P_i is the number of points counted for the tissue area of interest

ii. CE for Numerical Density

$$CE(N_v) = \sqrt{\frac{n}{n-1} \cdot \left[\frac{\Sigma(Q^-)^2}{\Sigma Q^- \cdot \Sigma Q^-} + \frac{\Sigma(P)^2}{\Sigma P \cdot \Sigma P} - \frac{2\Sigma(Q^- \cdot P)}{\Sigma Q^- \cdot \Sigma P}\right]}$$

where n is the number of sample points per area of interest, P is the total number of disectors falling in the area of interest and Q^- is the total number of cells counted (Cochran 1977).

iii. CE for Total Number

$$CE(N_{tot}) = \sqrt{CE^2(N_v) + CE^2(V_{ref})}$$

2.14.5 Validation of Stereological Methodology

Many scientists would like to see stereological techniques validated against previously used quantitative methods. There is an inherent problem with this since other quantitative assessments are potentially biased whereas stereological techniques known to be unbiased (Mayhew & Gundersen, 1996). However comparison with other 3-D techniques have shown that stereological methods yield accurate and biologically important data (Gundersen & Osterby, 1981; Rosen & Harry, 1990). This has lead to the increased use of stereological methods in biological sciences for quantitative estimations, for example, rat heart ventricle volume (Mattfedt, 1987), rabbit lung volume (Michel & Cruz-Orive, 1988), and total number of neurons in the human neocortex (Braendgaard *et al.*, 1990)

2.15 Full Blood Counts

In order to identify any systemic cellular changes occurring during PSA development or prevention with novel fibrin sealant full and differential cell counts were performed.

Routine human clinical full blood and differential cell counts have been performed by auto-analysers for many years due to the time intensive nature and potential inaccuracies of manual methods. The auto-analyser used was set up for human clinical investigations and hence potentially may produce inaccurate differential counts due to differences between human and rabbit blood cells which the auto-analyser was not configured to detect. Therefore to identify and eliminate incorrect results manual differential counts were performed in addition to auto-analysis.

2.15.1 Automated Counting

A Coulter MaxM (Coulter Electronics) was used for automated full blood counts. A 200 µm whole blood sample was aspirated by the analyser and separated into three parts for analysis:

i. Red blood cell (RBC) and platelet number determination

ii. White blood cell (WBC) number determination

Cell counts were all determined by the Coulter Principle. In summary, as cells pass through an aperture, across which an electric current flows, they interfere with that current. This interference is monitored electronically as pulses, with the number of pulses proportional to the number of cells and the magnitude of the pulse proportional to the volume of the cell.

2.15.2 Manual Differential Counting

Wedge blood films were made and stained using a Hema-Tek 200 staining machine (Ames, Bayers Diagnostics), which employs a modified Wright's stain (Appendix II). This, like Giemsa, is a Romanowsky stain and differentially stains cellular granules due to the binding of eosin and methylene blue.

Films were viewed under oil immersion with a final magnification of x1000. Caution was taken with regard to areas of the film analysed, with single cell layers chosen and avoidance of the tail end of the film which will contain a concentration of large cells due to the spreading motion during the preparation of the smear. Areas were systematically sampled in a tessellation motion and with a minimum of 100 cells counted for each sample .

2.16 Enzyme Linked Immunosorbent Assays (ELISAs)

To investigate any systemic and local biochemical modulation of PSAs and prevention with novel fibrin sealant, ELISAs for TNF-α and TGF-β were performed on blood and peritoneal fluid samples taken during the pathogenesis of PSAs and novel fibrin sealant

studies.

ELISAs employ antibodies to detect specific molecules and enzymes as sensitive and versatile amplification agents. In this way they provide an accurate detection system which is frequently utilised in medicine, for example, pregnancy tests.

Most ELISAs are based on a standard system. A microtitre plate is coated with an antibody directed against the molecule of interest (M). A standard or sample is then added, with the immobilised antibody capturing any molecules (M) present via an antibody-antigen reaction. The plate is then washed to remove any unbound substances and an antibody conjugated with an enzyme, directed against the molecule of interest (M) is added. This conjugated antibody binds via an antibody-antigen reaction to any bound molecule of interest (M). The plate is again washed to remove any unbound substances and a substrate is added to develop colour in proportion to the amount of conjugated antibody present and therefore the amount of molecule (M) present in the standard or sample. The colour reaction is stopped by acidification and the intensity of colour measured using a spectrophotometer, with sample concentrations of the molecule (M) determined from standard values (Figure 2.29).

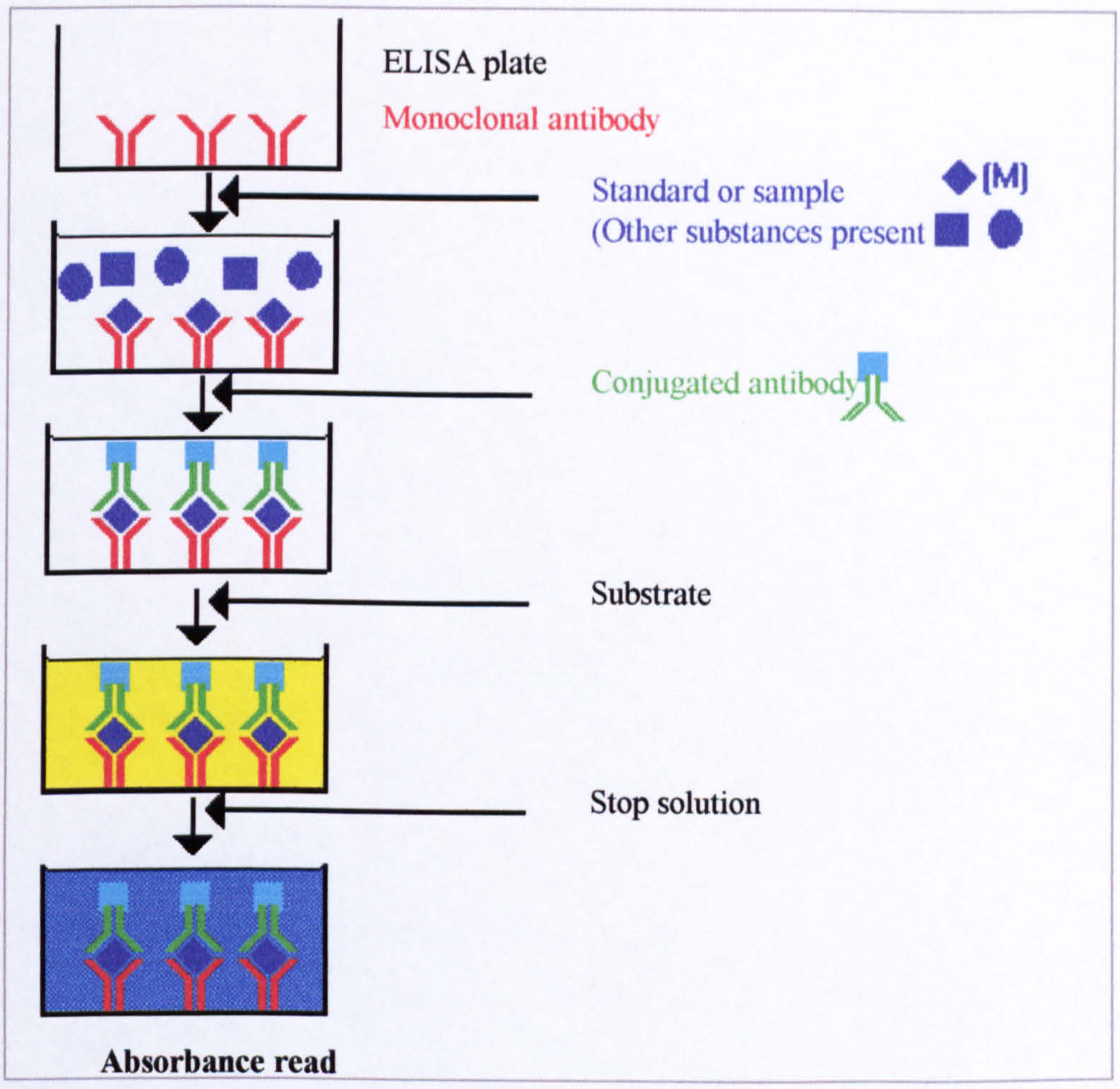


Figure 2.28: ELISA system.

2.16.1 Tumour Necrosis Factor-α ELISA (Genzyme)

(Genzyme Factor-Test-X™ Mouse TNF-α ELISA kit Protocol Booklet)

The Factor-Test-X™ Mouse tumour necrosis factor-α (TNF-α) ELISA kit (Genzyme Immunobiologicals, Cambridge, USA) was assessed for the detection of TNF-α in rabbit plasma and peritoneal fluid samples. It is a solid-phase ELISA employing the multiple antibody sandwich principle. The 96 well ELISA plate is pre-coated with monoclonal anti-mTNF-α and on addition of standards or samples, any TNF-α present is bound to the monoclonal antibody.

After washing the plate to remove any unbound material, a peroxidase-conjugated polyclonal anti-mTNF-α (HRP-conjugate) is added, which binds to the bound TNF-α. The plate is washed again to remove any unbound material. A substrate solution is then added which initiates a peroxidase catalysed colour change that is subsequently stopped

by acidification. The absorbance measured at 450nm is proportional to the concentration of TNF-α present in the standards or samples.

A standard curve is obtained by plotting the concentration of mTNF-α standards against their resulting absorbencies. The TNF-α concentration in experimental samples are then determined using the standard curve.

Validation

As described above the TNF-α ELISA kit used is intended for the detection of mouse TNF-α. Since this study was concerned with the measurement of TNF-α in rabbit plasma and peritoneal fluid samples, validation of the kit for the detection of rabbit TNF-α was required.

For this purpose a rabbit TNF-α control was necessary and since no rabbit TNF-α is available commercially, a control was produced by *in vitro* methods. Two methods were assessed for the production of rabbit TNF-α *in vitro* both using whole blood, the first isolating blood monocytes, the primary source of TNF-α, the second, which avoided monocyte isolation, used the whole blood sample (Appendix II).

Isolated monocytes or whole blood were cultured, together with lipopolysaccharide (LPS) solution, in 5% carbon dioxide at 37°C, to activate monocytes present to synthesise and release TNF-α. After the incubation period, the culture solution was collected, centrifuged to remove cellular or LPS components and the resulting solution containing any TNF-α collected and stored at -80°C.

Both rabbit and rat samples were used for obtaining TNF-α controls to determine the validity and reproducibility of the TNF-α production technique, since the ELISA kit used detects rat as well as mouse TNF-α.

Procedure (Genzyme Factor-Test-X™ Mouse TNF-α ELISA kit Protocol Booklet)

ELISA kit and samples were to brought up to room temperature, whilst the wash buffer (detergent in buffer) was prepared (1 wash buffer to 20 distilled water). TNF-α standards, 0, 35, 140, 560 and 2240pg/ml, were prepared by serial dilution of the supplied 8.96ng/ml TNF-α standard with diluted wash buffer.

All standards and samples were assayed in duplicate. 100µl of standard or sample was added to each well, with plasma samples diluted at least 1:2 with diluted wash buffer. The plate was incubated for 2 hours ± 5 minutes at 37 ± 2 °C. All wells were then aspirated and washed thoroughly 4 times using diluted wash buffer. 100µl of HRP-Conjugated anti-Mouse TNF-α was subsequently added to each well and incubated for 1 hour ± 5 minutes at 37 ± 2 °C.

Substrate solution was prepared by mixing equal volumes of Reagent A (0.001N hydrogen peroxide in buffered solution) and Reagent B (tetramethylbenzidine in methanol (<50%), DMSO (<2%) and glycerol (<15%)) no more than 15 minutes before use. Following incubation, wells were aspirated and washed thoroughly 4 times. 100µl of prepared substrate solution was added to each well and incubated for 10 ± 1 minutes at room temperature. 100µl of stop solution (1M sulphuric acid) was added to each well and stirred to ensure thorough mixing and homogenous colour. The plate was then read within 30 minutes using a plate reader (Dynatech MR7000) at 450nm.

TNF-α concentrations for samples were then calculated. All standards and sample absorbances were averaged and the blank reading (0 pg/ml TNF-α) was subtracted. A standard curve was plotted from the TNF-α standard results, with an order 1 regression curve fit used to read TNF-α concentrations for each sample. Dilution factors were taken into consideration in calculating absolute TNF-α concentrations for each sample.

2.16.2 Transforming Growth Factor-β_1 ELISA (Genzyme)

(Genzyme Predicta® TGF-β_1 ELISA Kit Protocol Booklet)

The Genzyme Predicta® Human Transforming Growth Factor-β_1 (TGF-β_1) ELISA Kit (Genzyme Immunobiologicals, Cambridge, USA) was assessed for the detection of rabbit TGF-β in collected plasma and peritoneal fluid samples.

The kit contains a 96-well microtitre plate containing immobilised mouse monoclonal antibody to TGF-β_1. For measurement of biologically active TGF-β_1, samples, standards and controls must be diluted and acidified, using hydrochloric acid (1N) for one hour. Following incubation, acidified samples and standards are neutralised to pH 7.0-7.4 with sodium hydroxide (1N), and are ready to be assayed or aliquotted and frozen until assay. After activation, standards ranging from 0.10 to 4.00ng/ml are prepared via serial dilution. However, final results of specimens must be calculated by multiplication by the appropriate dilution factor.

A measured volume of activated sample, standard, or control is added to each test well and incubated to allow any TGF-β_1 present to be bound by antibodies on the microtitre plate. The wells are then washed and a direct-labelled HRP-conjugated polyclonal antibody to TGF-β_1 is added which binds to the TGF-β_1 bound during incubation. After washing, a substrate solution is added to the wells, producing a blue colour in the presence of peroxidase. The colour reaction is then stopped by the addition of acid which changes the blue colour to yellow. The intensity of the yellow colour is directly proportional to the amount of TGF-β_1 present in the sample, standard, or control. The absorbance of each well is read at 450nm and a standard curve is constructed to quantitate TGF-β_1 concentrations in the controls and samples.

Validation

The ELISA kit used for the detection of rabbit TGF-β is intended for the detection of human TGF-β_1, hence validation of the kit with regard to its detection of rabbit TGF-β was carried out.

There is no commercially available rabbit TGF-β hence rabbit TGF-β controls were

produced *in vitro* (Appendix II). Whole blood samples were taken from both rabbit and human subjects, with the human sample determining the validity and reproducibility of the TGF-β preparation technique. Samples were centrifuged for separation of platelet rich plasma, with platelets being the primary source of TGF-β. This platelet rich plasma was further centrifuged to obtain a platelet concentrate, with a small volume of platelet poor plasma used to resuspend cells. Platelets were then cytolysed in order for stored TGF-β to be released using a freeze (-80°C)/thaw cycle and the TGF-β rich substrate collected and either assayed immediately or stored (-80°C).

Procedure (Genzyme Predicta®Human TGF-β_1 ELISA kit Protocol Booklet)

All samples and the ELISA kit were allowed to reach room temperature before the assay was commenced. Appropriate volumes of wash buffer (concentrated detergent solution with 0.02% thimerosal as preservative) were prepared, using a 20 fold dilution of wash reagent to distilled water.

Standard and sample were first diluted by adding 20µl of TGF-β_1 standard to 440µl of sample diluent (buffered solution with protein and <0.01% Proclin-300 as preservative) or by adding 10 µl of plasma sample to 450µl of sample diluent. Standards and samples were activated by addition of 20µl of 1N hydrochloric acid, gently mixed and then incubated at 2-8°C for 60±2 minutes. Neutralisation then occurred by adding 20µl of 1N sodium hydroxide and mixing.

Serial dilutions of the activated TGF-β_1 stock standard (4ng/ml) with sample diluent resulted in TGF-β_1 standards of 0, 0.10, 0.26, 0.64, 1.60 and 4.00ng/ml.

100µl of activated standard or sample was added to each well and incubated for 60±2 minutes at 37±2 °C. Wells were then aspirated and washed well 5 times. 100µl of anti-TGF-β_1 HRP-conjugate was then added to each well and incubated for 60±2 minutes at 37±2 °C. Wells were again aspirated and washed 5 times.

100µl of substrate solution (tetramethylbenzidine (<2%) in solvent (<40%) and 0.03% hydrogen peroxide in buffered solution) was added to each well and allowed to incubate for 20±1 minutes at room temperature. 100µl of "stop" solution (acid solution (<2N)) was then added to each well, which was stirred and absorbance read at 450nm within 30 minutes using a plate reader (Dynatech MR7000).

To calculate the concentration of TGF-β_1 in each standard and sample, the mean was calculated from duplicate readings and the blank reading was subtracted. A standard curve was produced (regression order 1) and TGF-β_1 concentrations for each sample read off the standard curve. Multiplication by the dilution factor then resulted in the absolute TGF-β_1 concentration for each sample.

2.16.3 TGF-β_1 ELISA (R& D Systems)

(R&D Systems Quantikine™ Human TGF-β_1 Immunoassay Protocol Booklet)

The Quantikine™ Human TGF-β_1 Immunoassay (R&D Systems, Abingdon, UK) employs the quantitative sandwich ELISA technique, and was used for analysis of plasma and peritoneal fluid sample for TGF-β_1 content.

TGF-β soluble receptor Type II which binds TGF-β_1 has been pre-coated onto a microtitre plate. Standards and samples are pipetted into the wells and any TGF-β_1 present is bound by the immobilised receptor. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF-$\beta_1$is added to the wells to sandwich the TGF-β_1 bound during the first incubation. The soluble receptor binds TGF-β_1, TGF-β_3, and TGF-β_5 with similar affinities. The sensitivity of the immunoassay is provided by the polyclonal antibody which is specific for TGF-β_1. The soluble receptor also binds TGF-β_2 but with an affinity at least 100 fold lower. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of TGF-β_1 bound in the initial step. The colour development is stopped by acidification and the intensity of the colour is

measured using a spectrophotometer.

Validation

The same technique was used as described in 2.16.2.

Procedure (R&D Systems Quantikine™ Human TGF-β_1 Immunoassay Protocol Booklet)

All samples and the ELISA kit were brought up to room temperature prior to initiation of the assay.

Samples required activation before analysis. 100µl of plasma was added to 100µl 2.5N acetic acid/10M urea solution, mixed well and incubated for 10 minutes at 25°C. 100µl of 2.7N sodium hydroxide/1M HEPES solution was then added and mixed well to stop the activation process. During the activation process samples underwent a 3 fold dilution. Samples were then further diluted 4 fold with calibrator diluent RD6M (animal serum with preservative). Hence overall samples were diluted 12 fold.

The appropriate amount of wash buffer (concentrated solution of buffered surfactant with preservative) was prepared in a 1:25 fold dilution with distilled water. TGF-β standard was reconstituted by addition of 2ml of Calibrator Diluent RD6M to TGF-β standard vial. The resulting concentration of standard stock solution was 2000pg/ml. TGF-β standards, 0, 31.2, 62.5, 125, 250, 500, 1000 and 2000pg/ml, were then prepared by serial dilution of the stock standard.

All standards and samples were assayed in duplicate. 200µl of standard or sample was added to each well and incubated for 3 hours at 25°C. After which each well was aspirated and washed thoroughly 3 times, 200µl of Conjugate was added to each well and incubated for 1.5 hours at 25°C.

Substrate solution was prepared by mixing equal volumes of Substrate A (stabilised

hydrogen peroxide) and Substrate B (stabilised chromogen (tetramethylbenzidine)).

Following the incubation period all wells were aspirated and washed thoroughly 3 times. Then 200µl of prepared substrate solution was added to each well and incubated for 20 minutes at 25°C. Finally 50µl of stop solution (2N sulphuric acid) was added to each well and mixed well to ensure even colour distribution. Plates were read on a plate reader (Dynatech MR7000) plate reader at 450nm within 30 minutes.

Calculation of TGF-β concentration for each sample was carried out using the TGF-β standard results. All standard and sample absorbance readings were averaged and the mean blank reading (0 pg/ml TGF-β) subtracted from each reading. A standard curve was produced and samples values were read off the order 1 regression of the curve. Finally, dilution factors were used to calculate the actual TGF-β concentrations for each sample assayed.

2.17 Statistical Analysis

All data was collected into respective experimental groups, with mean, standard deviation, standard error of the mean and percentage coefficient of variation calculated using MS Excel 5.0.

Statistical comparisons between experimental groups was performed using Student's t test. In cases where normality or equal variance failed, Mann Whitney Rank Sum test was used. All tests were performed using the computer package Sigma Stat 1.0.

3.0 Pathogenesis of PSAs

3.1 Introduction

Interest in post surgical adhesions (PSAs) has been prevalent since the advent of surgery. Their effects on patients health, economic and workload burdens have resulted in much research investigating potential PSA preventative agents, ranging from inabsorbable barriers to fibrinolytic agents (refer to Section 1.2). However despite much effort there currently is no commercially available PSA preventative agent.

It has been hypothesised by both this and other authors that this lack of success is primarily attributed to many aspects of PSA development being undetermined. Although comprehensive qualitative studies investigating PSA formation were conducted in the 1960-70's, little research in this area has occurred since with studies concentrating on potential anti-PSA agents. Hence there is a lack of quantitative knowledge available regarding the physiological and biochemical mechanisms governing PSA development. In order for potential PSA preventative agents to be optimised for successful PSA prevention further understanding of the mechanisms controlling PSA development is a necessity.

The aims of this study were to:

i. quantitatively define physiological, structural, cellular and biochemical mechanisms occurring during the development of PSAs using design based, unbiased, stereological techniques in an established experimental model
ii. identify possible windows of pathogenesis where pharmaceutical intervention may be most effective.

3.2 Materials and Methods

A rabbit uterine horn abrasion model was used to induce peritoneal PSAs (surgical and termination procedures described in Chapter 2). Defined areas on the uterine horn and corresponding peritoneal wall were abraded using a standard template and scraper. These

injured areas were held in juxtaposition via positional sutures located outside the experimental site and timing commenced from this point. Tissues were sampled at 30 seconds, 1, 3, 5, 10, 30 minutes, 1, 4, 8, 16 hours, 1, 3, 5, 7, 14, 28 and 42 days following injury, with each time point group consisting of 8 experimental sites.

Resected tissues (experimental sites) were uniform randomly sliced transversely (refer to Section 2.9). Odd numbered slices were processed to paraffin wax for volumetric analysis (tissue zones and structural changes) and even numbered sections processed to resin, for number density analysis for cellular involvement (Chapter 2).

No morbidity or mortalities were observed throughout this study.

3.3 Macroscopical Analysis

General PSAs associated with laparotomy were seen in all cases, that is, PSAs from the peritoneal suture line to underlying tissues, including the omentum, fat pad over the bladder and the bladder itself. None of these PSAs interfered with any of the experimental sites.

Within as little as 5 minutes following injury fibrin strands could be seen between the abraded surfaces of the uterine horn and peritoneal wall. By 30 minutes the two surfaces were adhered along their length *in vivo* with this trend continuing from there on (Figures 3.1 and 3.2). The overall macroscopic incidence of PSAs *in vivo* from 10 minutes and onwards was 90%, with a detailed breakdown shown on Graph 3.1 (Appendix III Table 3.1).

Experimental tissues were carefully resected for microscopical analysis. Since experimental sites were 100% adhered by 10 minutes post injury, their macroscopic appearance throughout development showed little change (Figures 3.1 and 3.2).

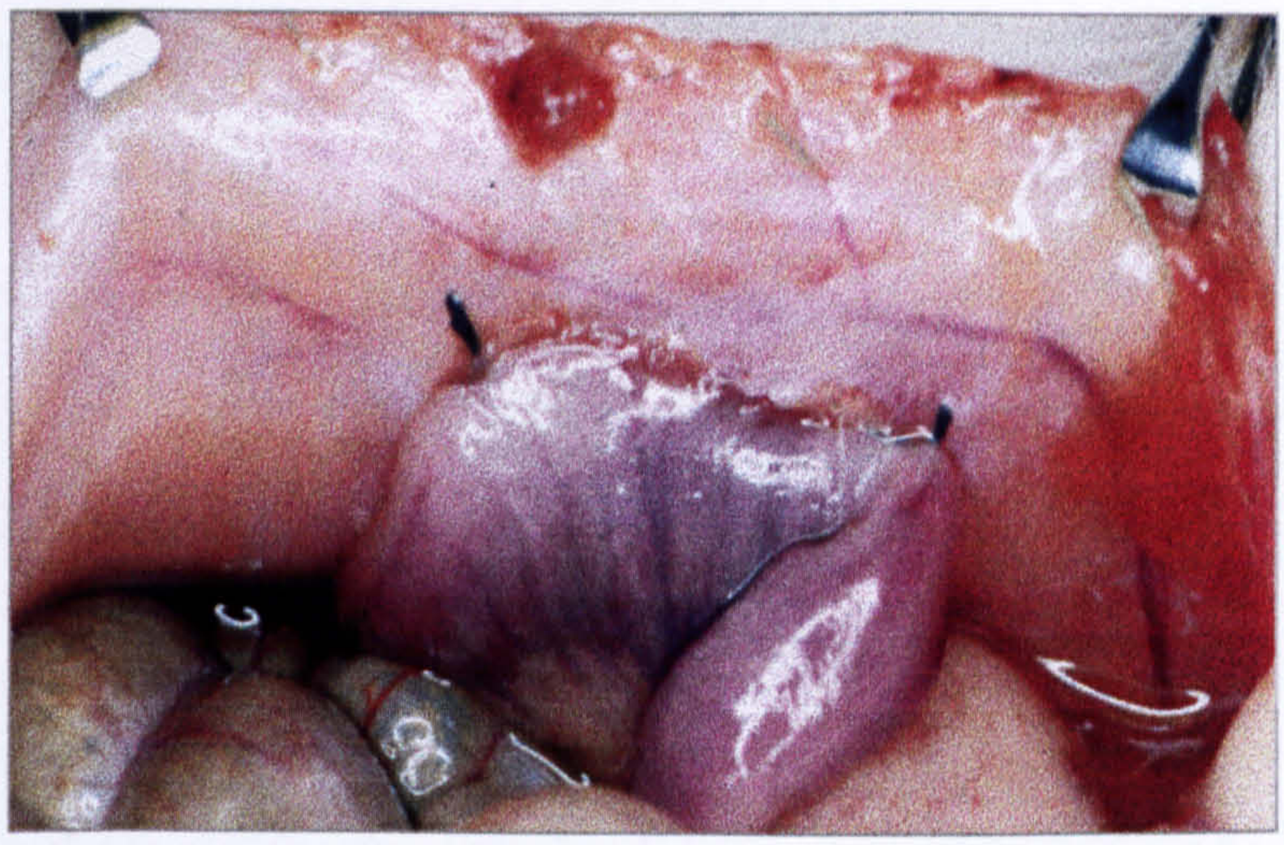


Figure 3.1: Macroscopic PSA at 3 days post injury.

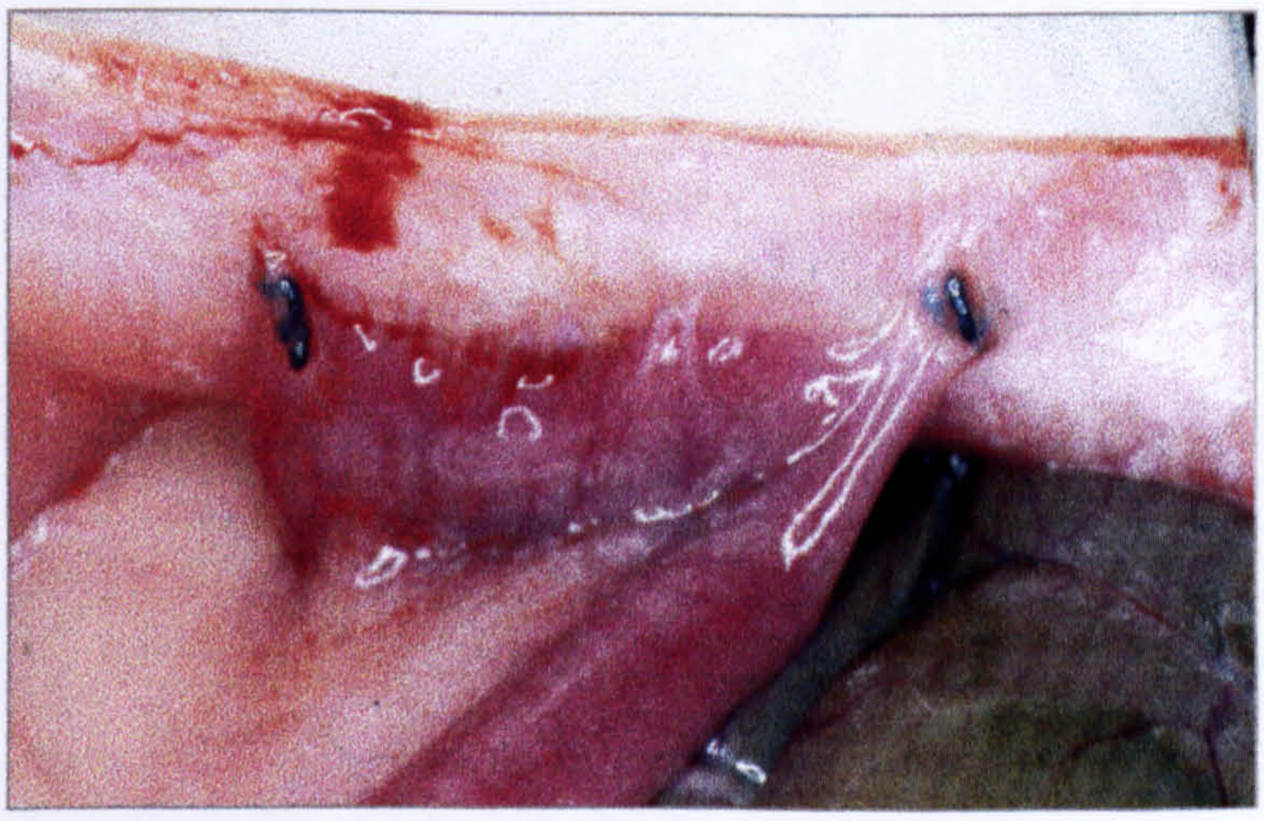
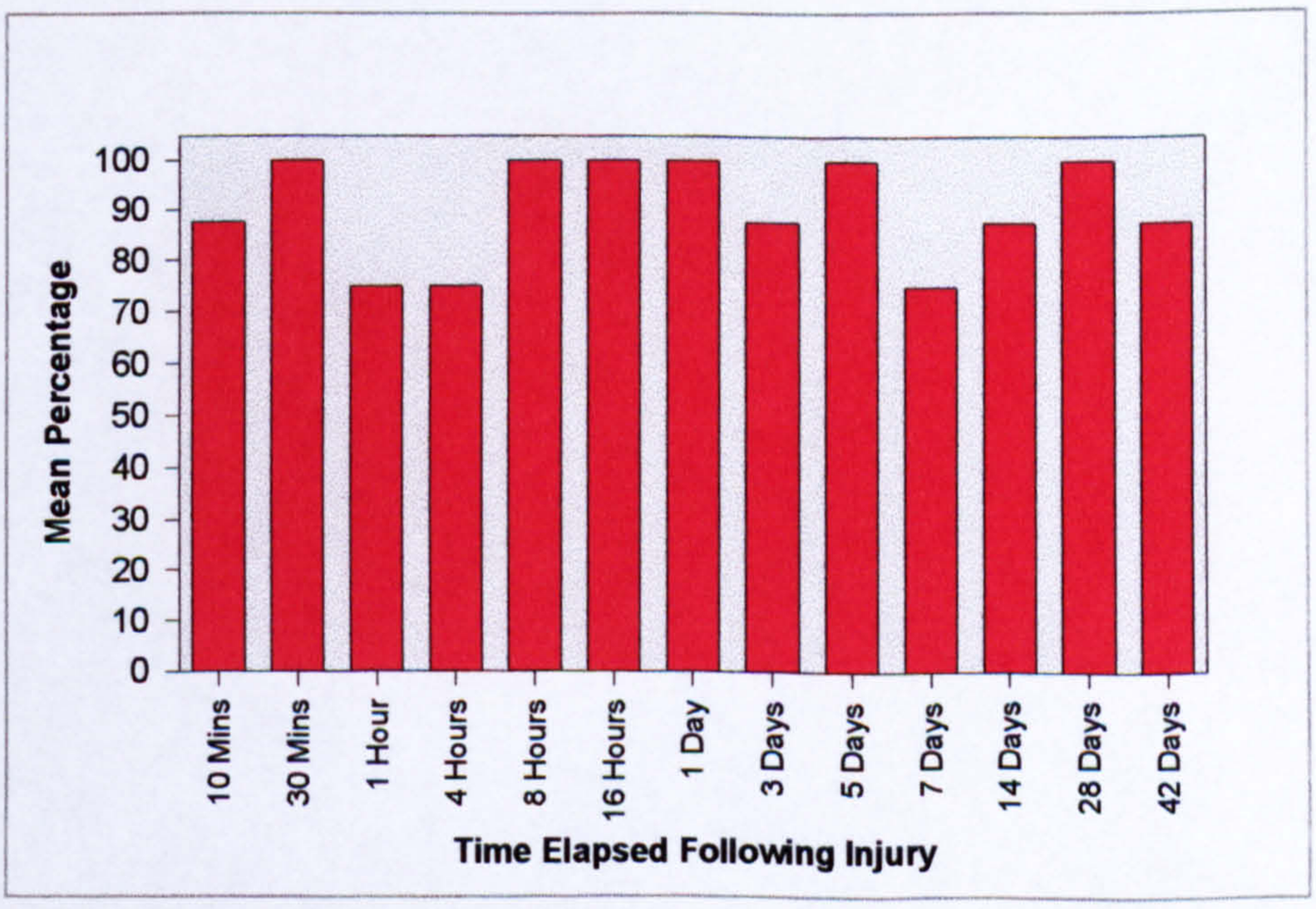


Figure 3.2: Macroscopic PSA at 14 days post injury.



Graph 3.1: Mean macroscopic incidence of PSAs.

3.4 Volumetric Analysis of Tissue Zones

It is universally recognised that PSA formation is a complex, multi-factorial process. In order to understand the basic physiological changes occurring during PSA development quantitative analysis of different tissue components was performed for all experimental sites. Hence physiological changes occurring in PSAs from 30 seconds to 42 days were investigated.

Odd numbered uniform random slices from each experimental site were processed to paraffin wax and sections cut at 5μm. One section from each block was stained with haematoxylin and eosin and analysed using Cavalieri's Principal. Absolute volumes of PSA, peritoneal and uterine horn tissue generation and damage zones, peritoneal reaction and cell concentration zones and uterine horn haemorrhage (Figure 3.1) were calculated for each experimental site, with mean volumes subsequently determined for each experimental time point (refer to Chapter 2).

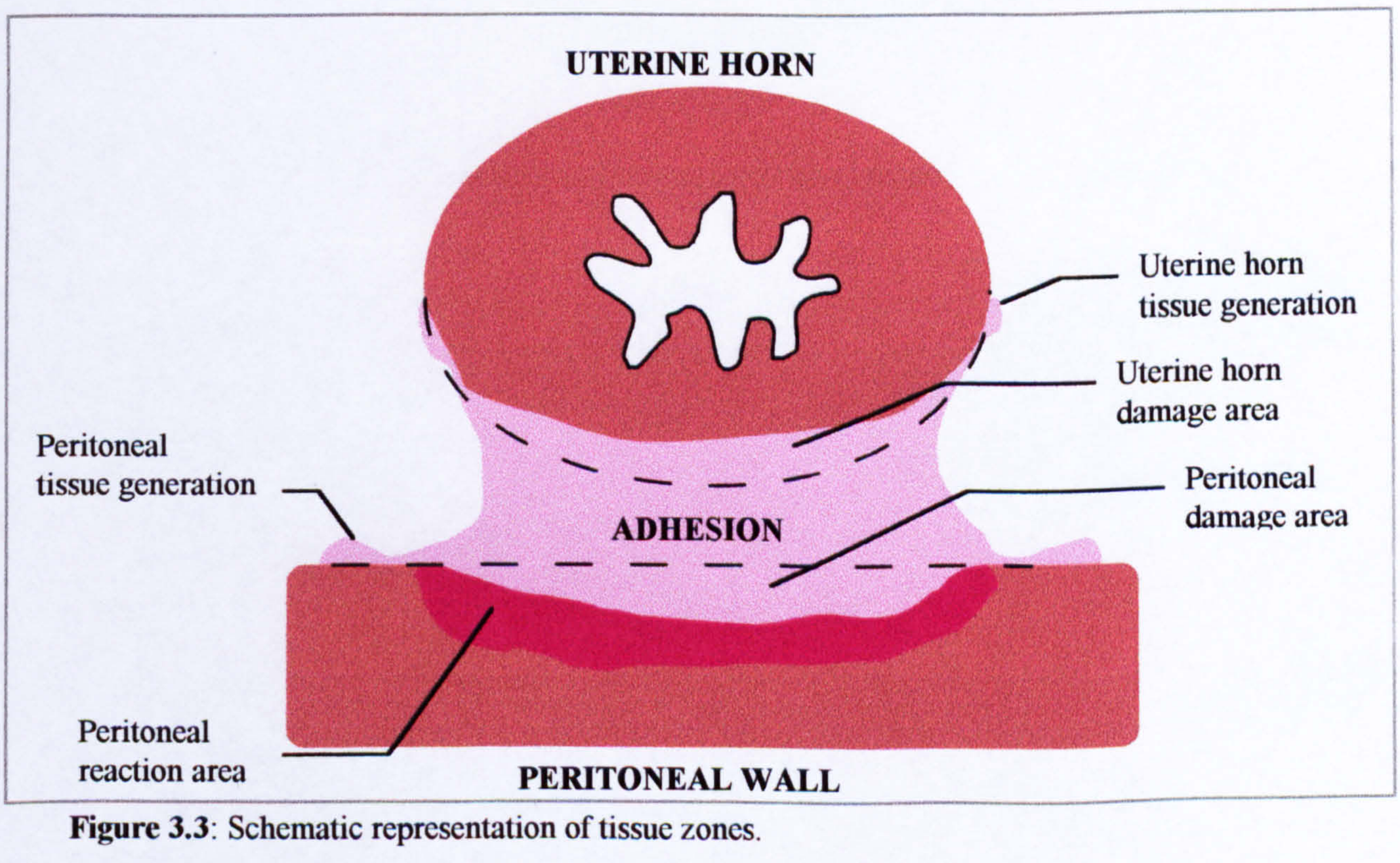
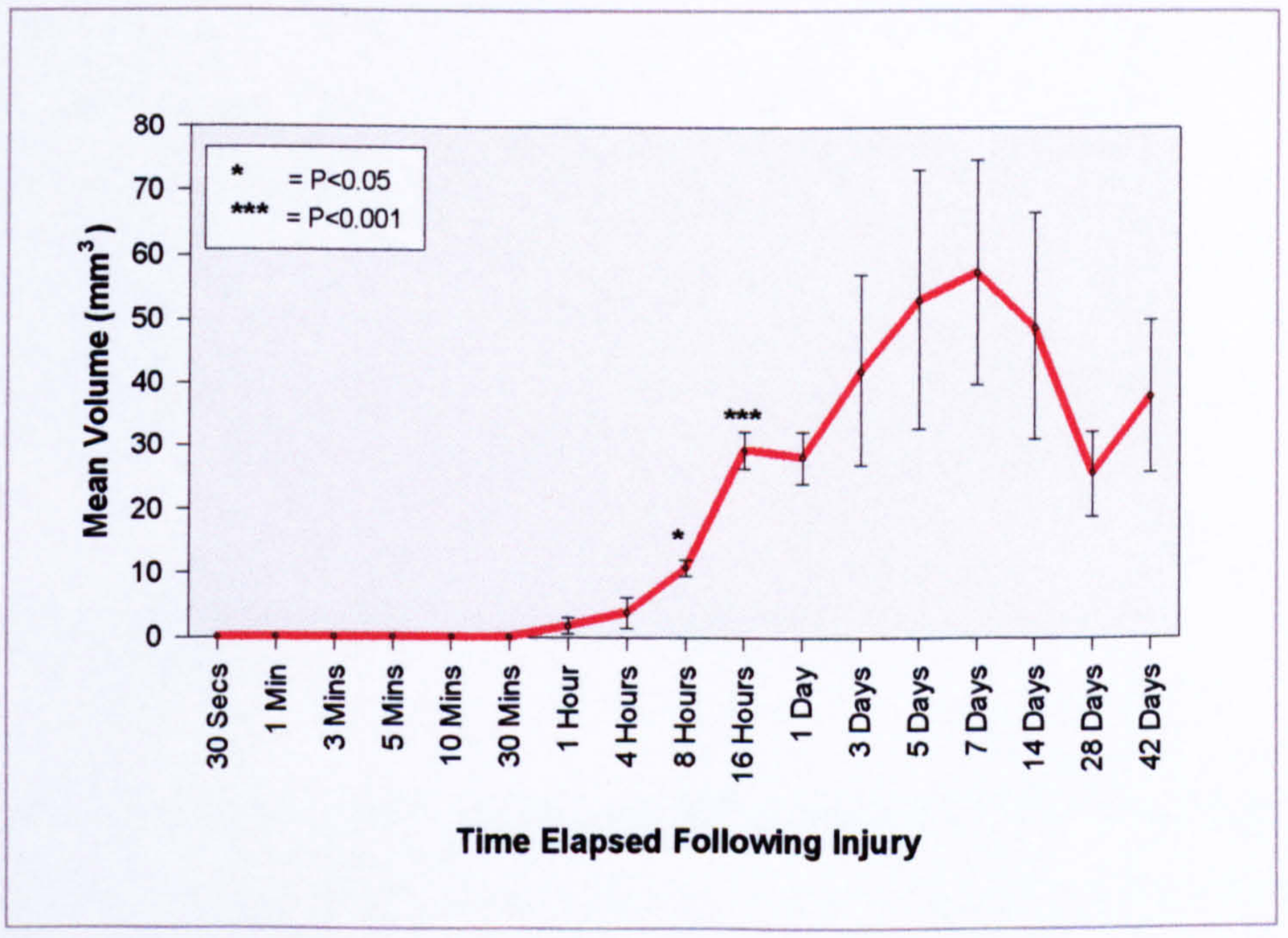


Figure 3.3: Schematic representation of tissue zones.

3.4.1 Results

Microscopically, PSAs were first evident 1 hour following injury and at all subsequent time points (Graph 3.2; Appendix III Table 3.2; Figure 3.4). Mean PSA volume

increased significantly ($P<0.05$) up to 16 hours following, with the most rapid increase occurring between 4 and 16 hours (Figure 3.5). Up to 7 days a trend for mean PSA volume to increase was observed, after which there was a trend to decrease, although not statistically significant ($P>0.05$) time point to time point (Appendix III Table 3.3).



Graph 3.2: Mean PSA volumes
together with standard error of the means (SEMs) and statistical differences.

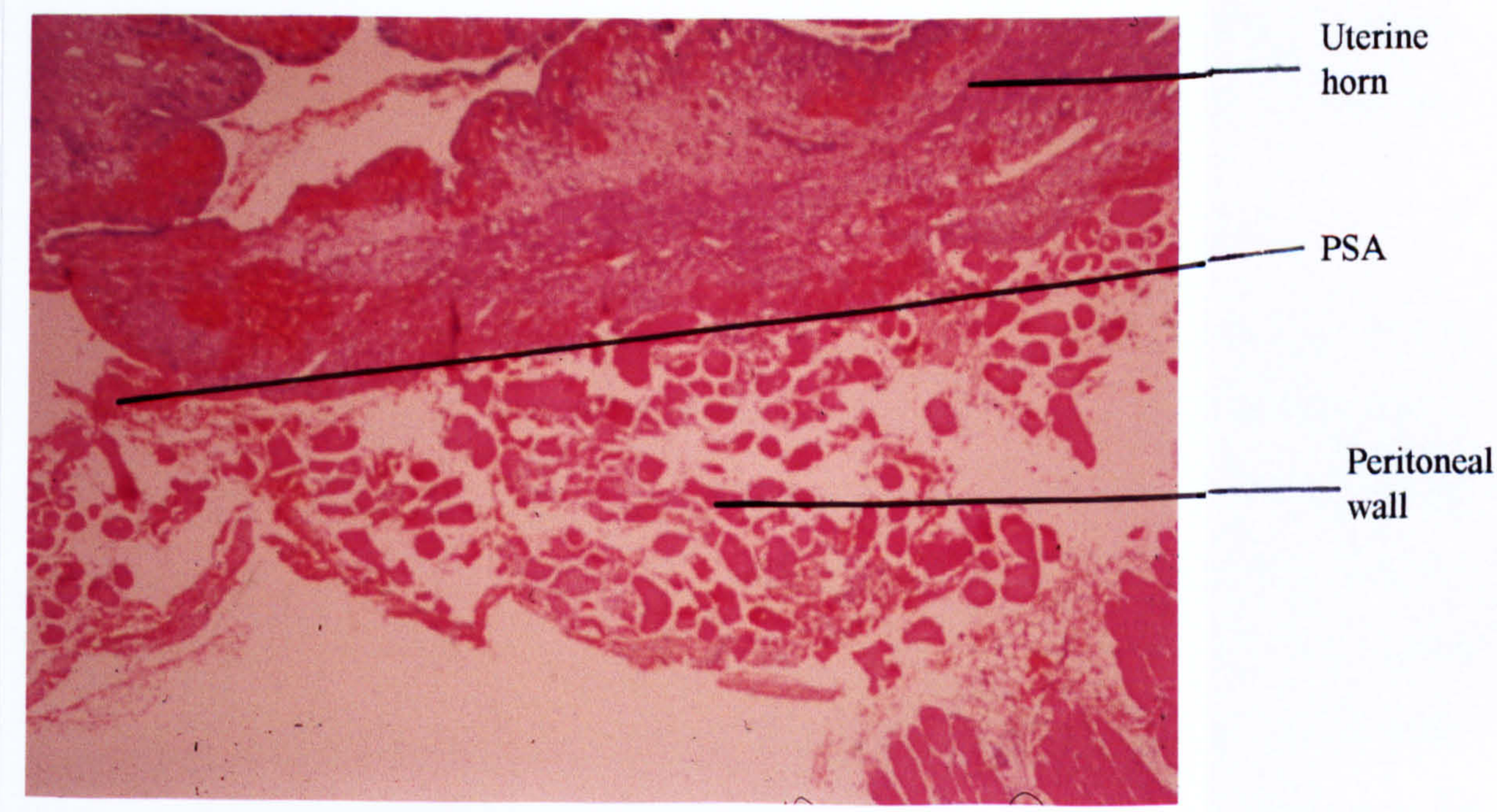


Figure 3.4: 1 Hour PSA
(M_o x2)

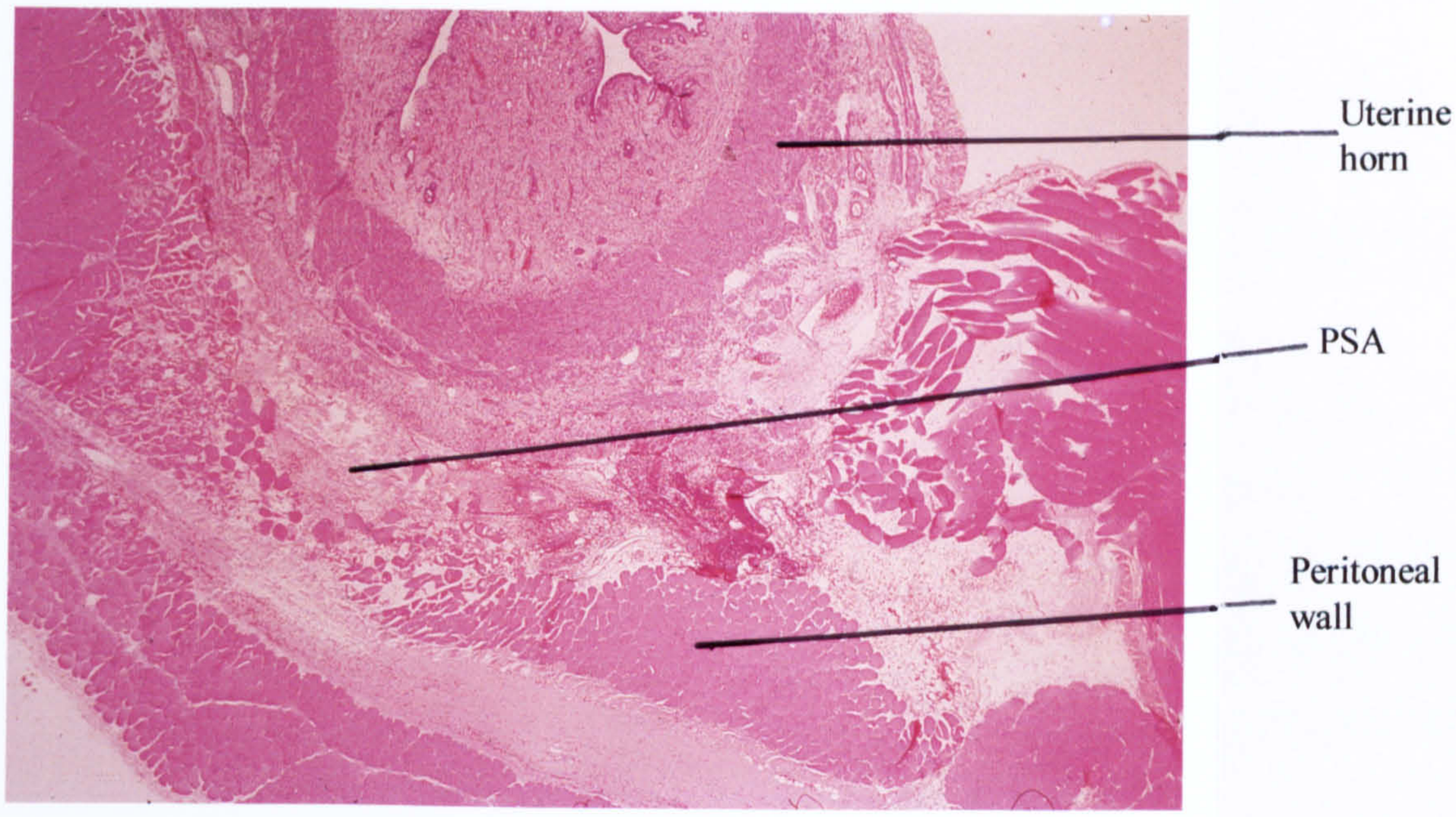


Figure 3.5: PSA development at 16 hours post injury (M_o x2)

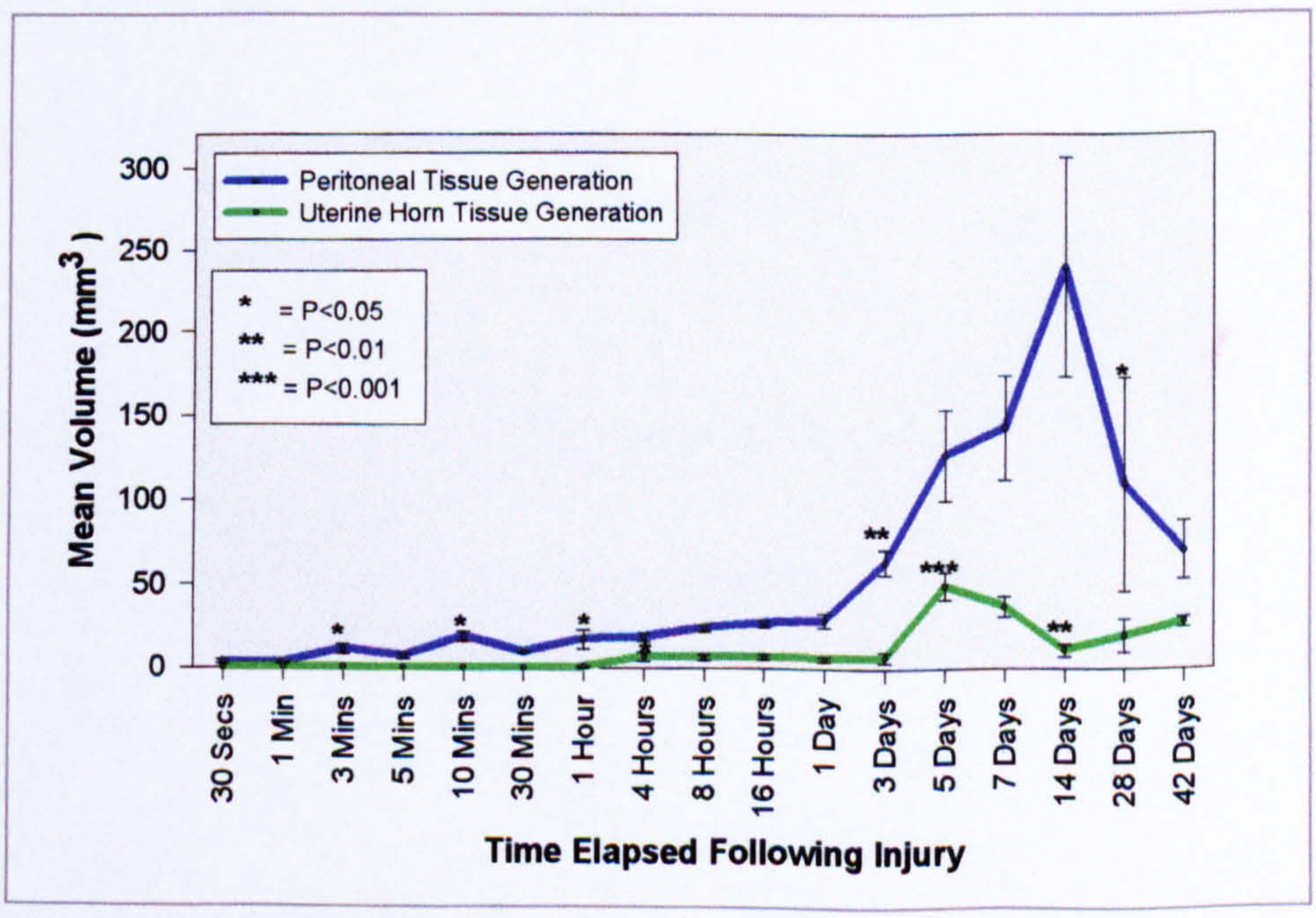
Peritoneal tissue generation (PTG) was present at all sampling time points (Graph 3.3; Appendix III Table 3.2). From 30 seconds to 1 day post injury mean volume of PTG showed a gradual trend to increase. A significant increase ($P<0.01$) in mean volume was seen between 1 and 3 days. From 3 to 14 days a trend of slower increase in mean volume was observed (Figure 3.6), followed by a significant decrease ($P<0.05$) between 14 and 28 days, with volume continuing to decrease but at a much slower rate at 42 days post injury (Appendix III Table 3.3).

Uterine horn tissue generation (UTG) was first seen microscopically after 1 hour and at all time points from there on (Graph 3.3; Appendix III Table 3.2). An overall increase in mean volume was seen between 1 hour and 5 days. Significant increases ($P<0.05$) were seen between 1 and 4 hours and between 3 and 5 days ($P<0.001$) (Figure 3.7). From 5 days post injury a trend to decrease was seen, with a significant decrease ($P<0.01$) seen between 7 and 14 days. After 14 days there was a trend for mean volume to increase, although this was not significant ($P>0.05$) from time point to time point (Appendix III Table 3.3).

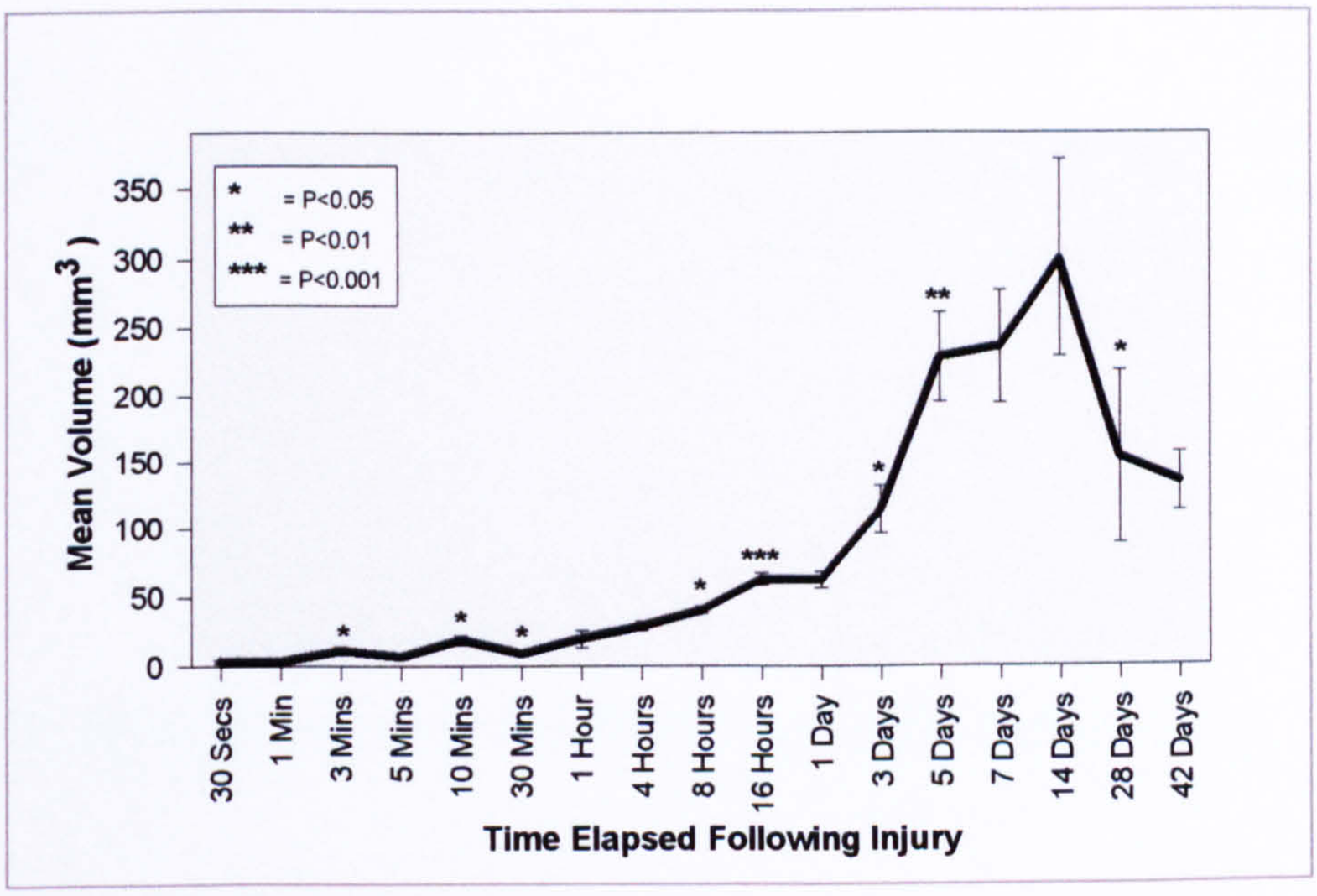
Mean volume of total tissue generated (TTG) demonstrated a trend to increase up to 14 days post injury with significant increases seen at 3 and 20 minutes, 8 and 16 hours, 3 and 5 days (Graph 3.4; Appendix III Tables 3.2 and 3.3). Following the peak in mean volume a significant decrease is observed at 28 days, after which little change occurred.

Mean volume for experimental sites for peritoneal damage was found to be fairly constant from 30 seconds to 3 days (Graph 3.5; Appendix III Table 3.2). From 3 to 5 days there was a significant increase ($P<0.01$) in mean volume, followed by a gradual increase (non-significant) up to 14 days (Figure 3.6). Following this a sharp decline was observed although this was not statistically significant ($P>0.05$) time point to time point (Appendix III Table 3.3).

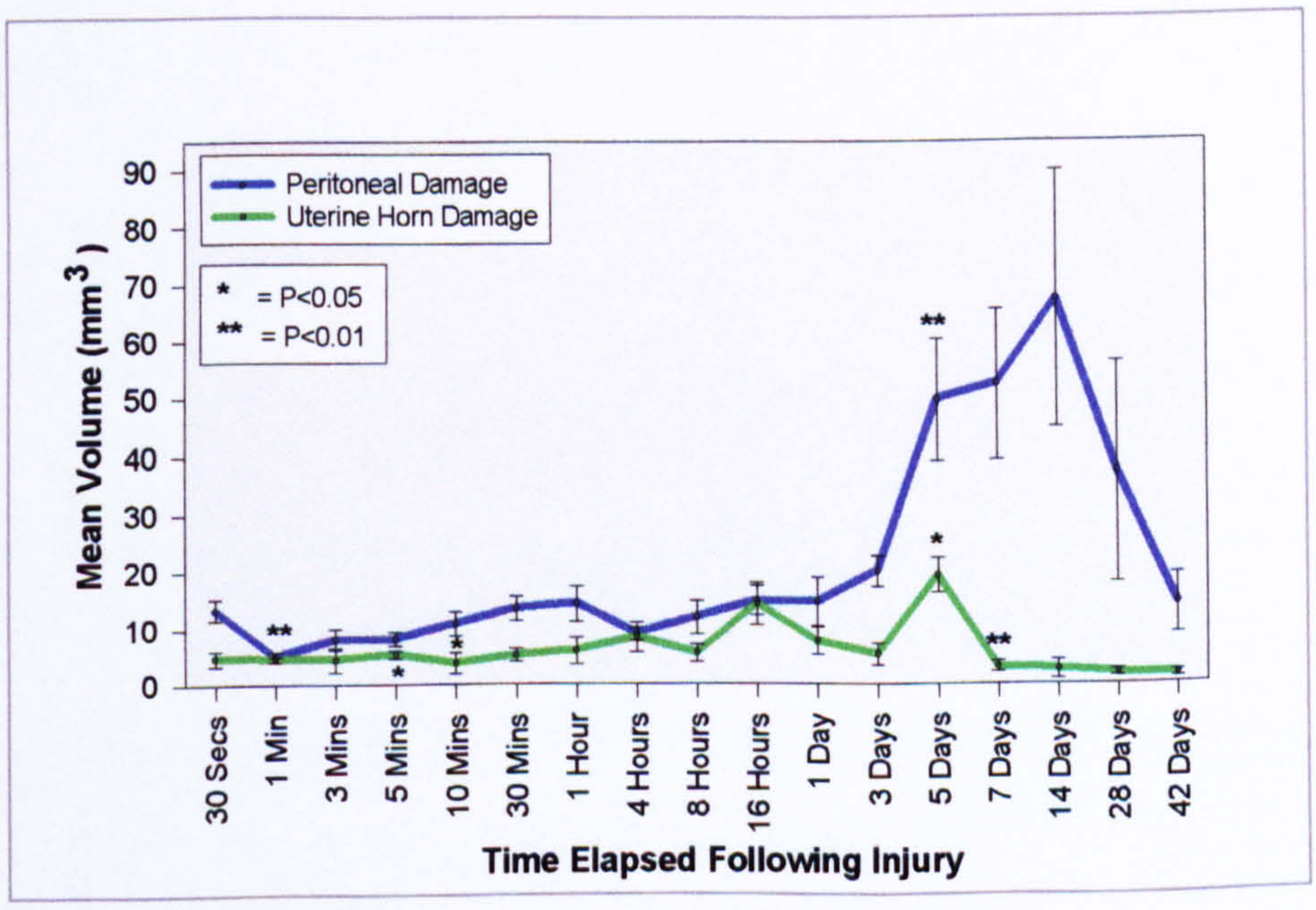
Uterine horn damage showed an overall constant mean volume per injury site from 30 seconds to 3 days (Graph 3.5; Appendix III Table 3.2). A significant increase ($P<0.01$) occurred between 3 and 5 days (Figure 3.7). Between 5 and 7 days a significant decrease ($P>0.001$) in mean volume was seen (Appendix III Table 3.3). From 7 days to the end of our study mean volume of uterine horn damage remained constant.



Graph 3.3: Mean tissue generation volumes
together with SEMs and statistical differences.



Graph 3.4: Mean TTG volumes
together with SEMs and statistical differences.



Graph 3.5: Mean damage volumes
together with SEMs and statistical differences.

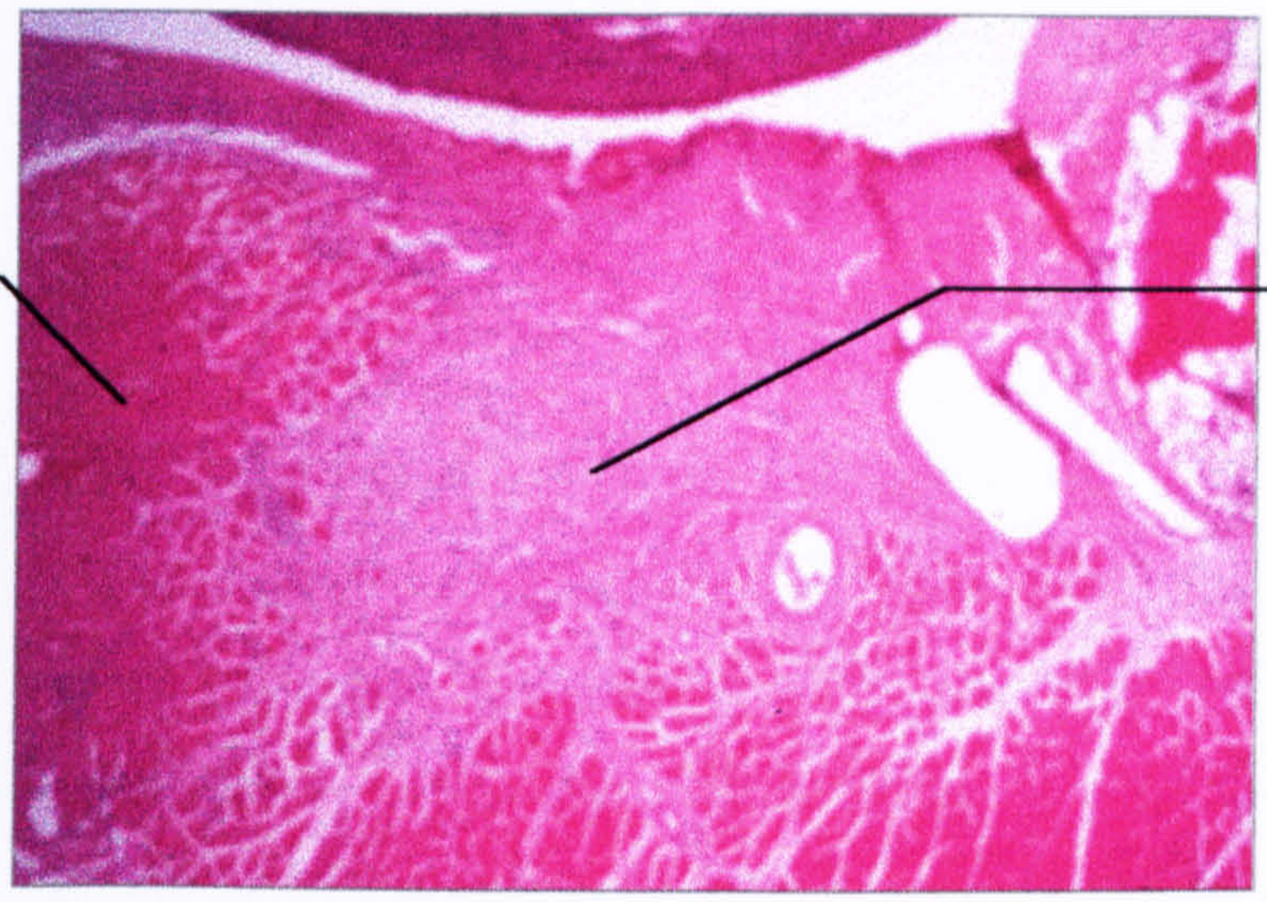


Figure 3.6: Peritoneal damage and tissue generation at 14 days post injury. (M_o x2)

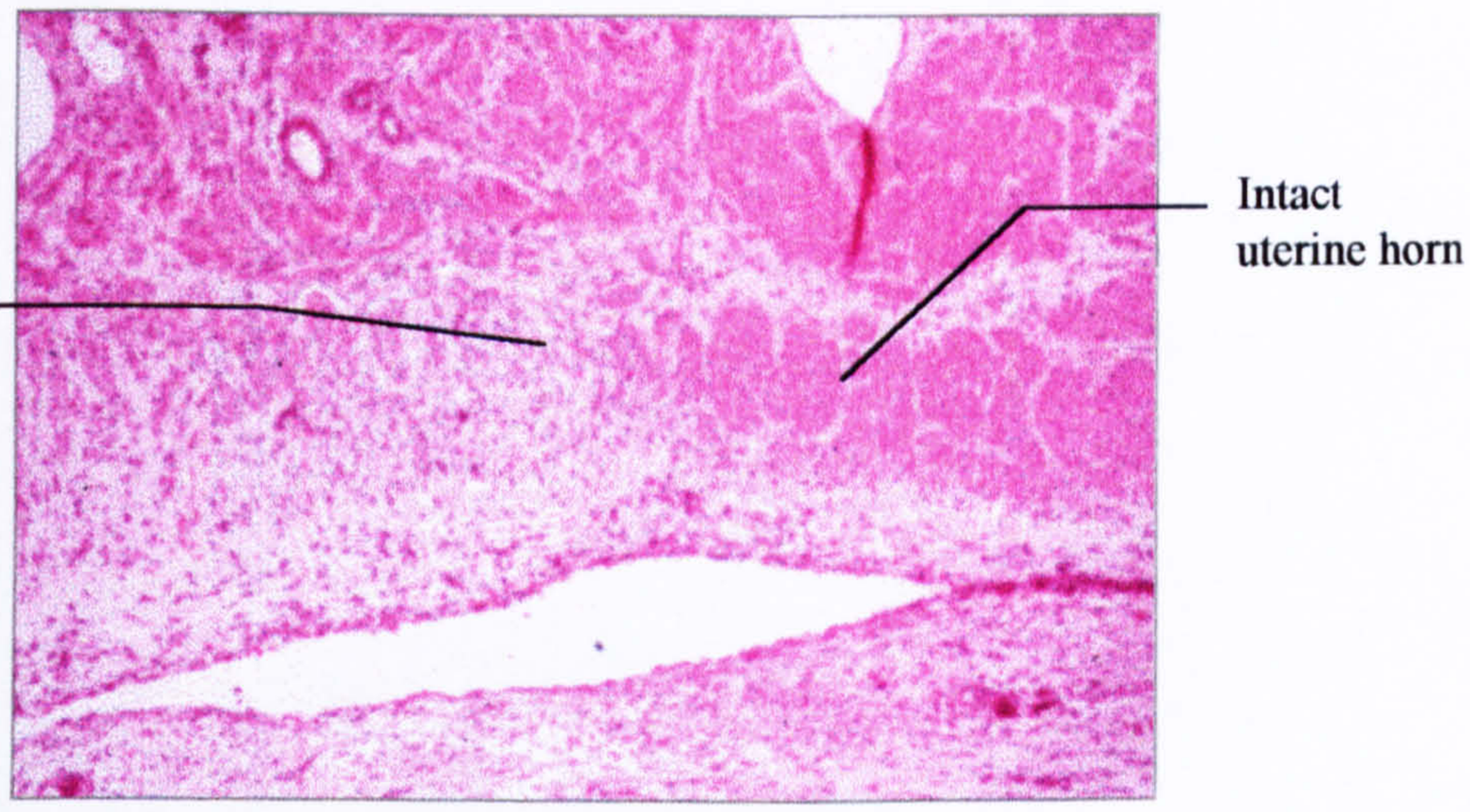


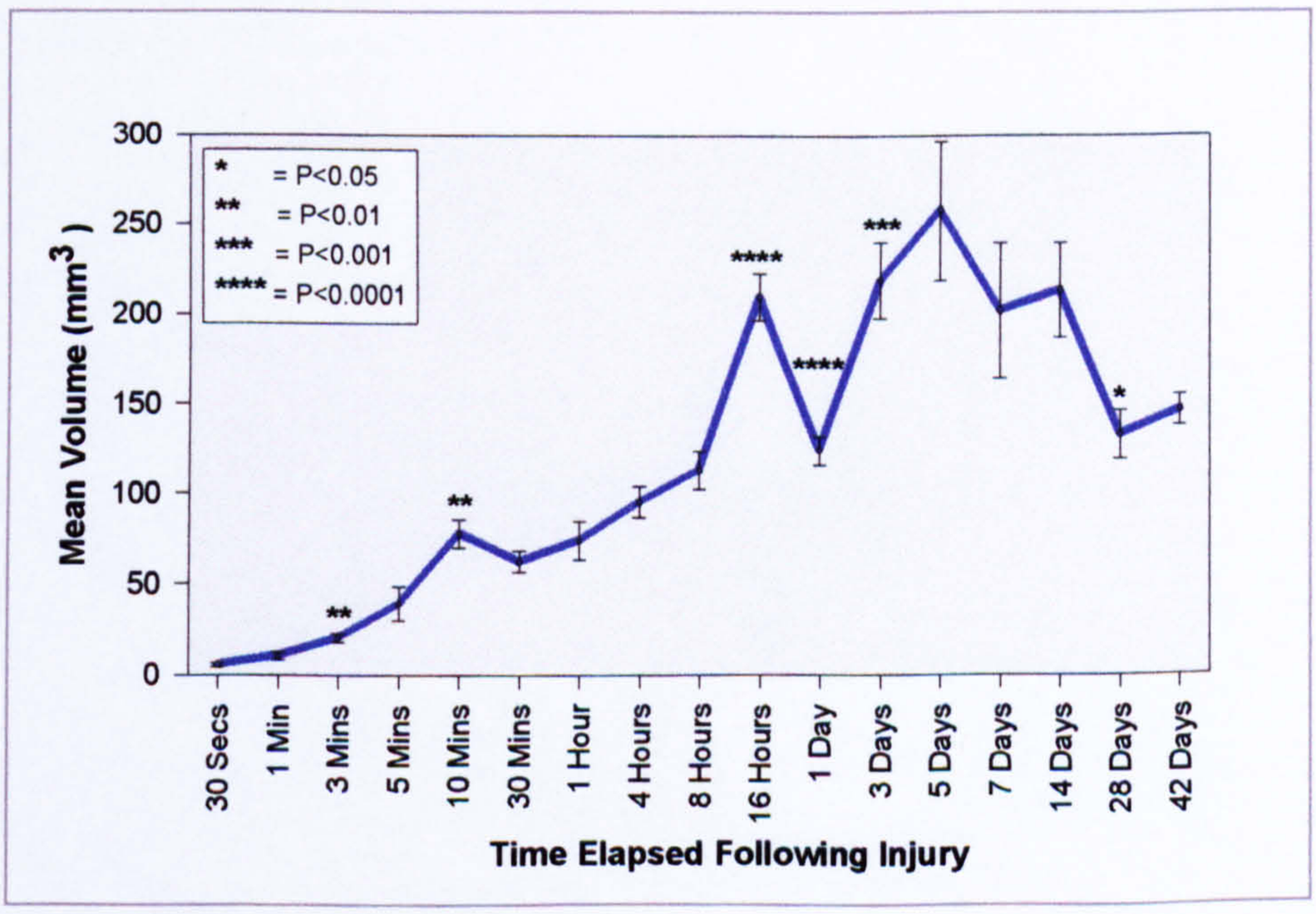
Figure 3.7: Uterine horn damage and tissue generation at 5 days post injury. (M_o x10)

Peritoneal reaction was evident at 30 seconds after injury (Graph 3.6; Appendix III Table 3.2) and demonstrated a steady increase in mean volume up to 8 hours. From 8 to 16 hours there was a significant increase ($P<0.0001$) in mean volume, then a significant decrease ($P<0.001$) from 16 hours to 1 day. This was followed by a significant increase ($P<0.001$) from 24 hours to 3 days with mean volume remaining constant between 3 and 5 days. After 5 days there was a steady decrease in mean volume per time point group (Appendix III Table 3.3).

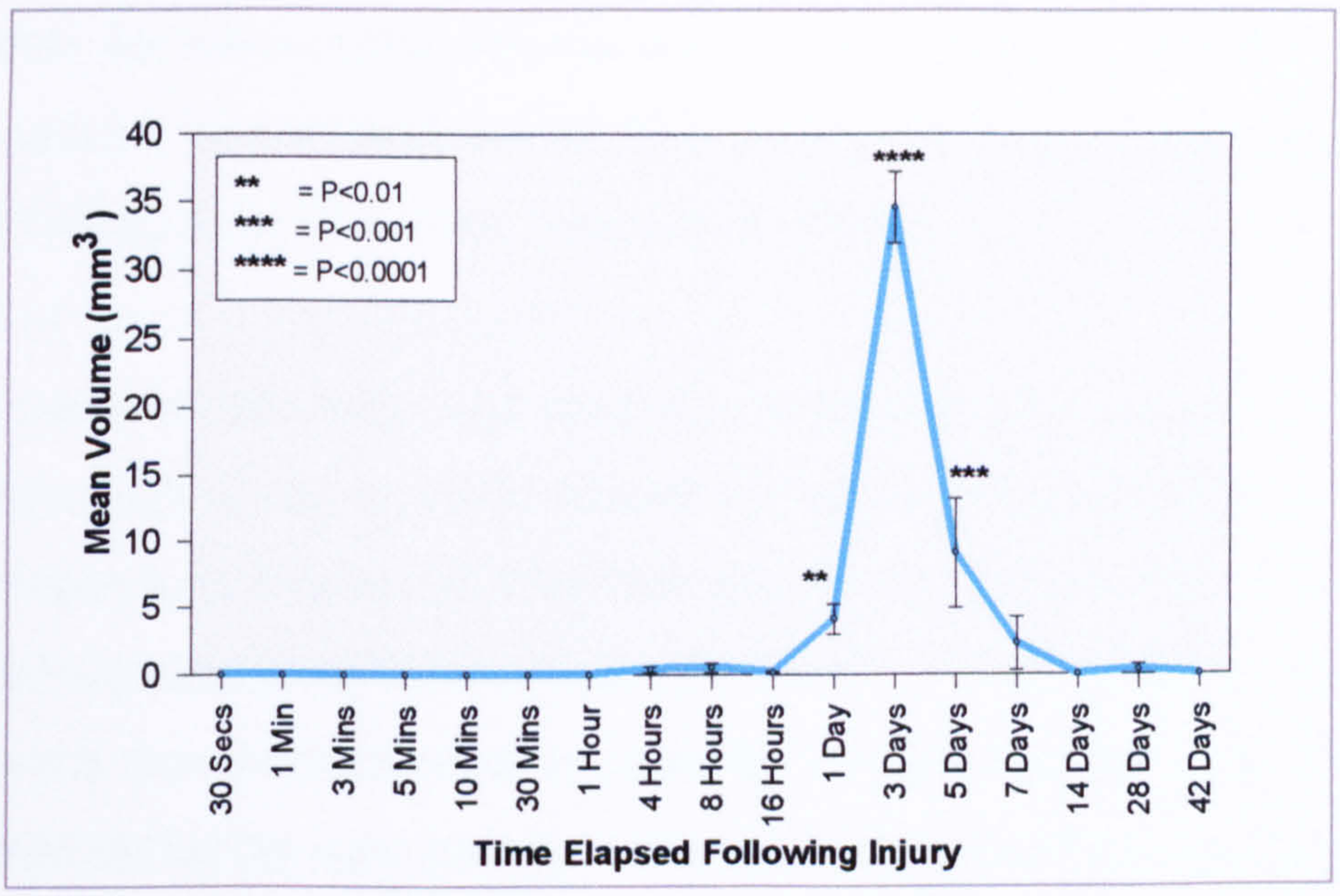
Peritoneal cellular concentration was not present until 4 hours after injury, and levels seen up to 1 day were minimal (Graph 3.7; Appendix III Table 3.2). After 16 hours a

significant increase ($P<0.05$) in mean volume was observed which peaked at 3 days. Following this was an equally sharp decrease, with non detectable levels seen from 14 days onwards (Appendix III Table 3.3).

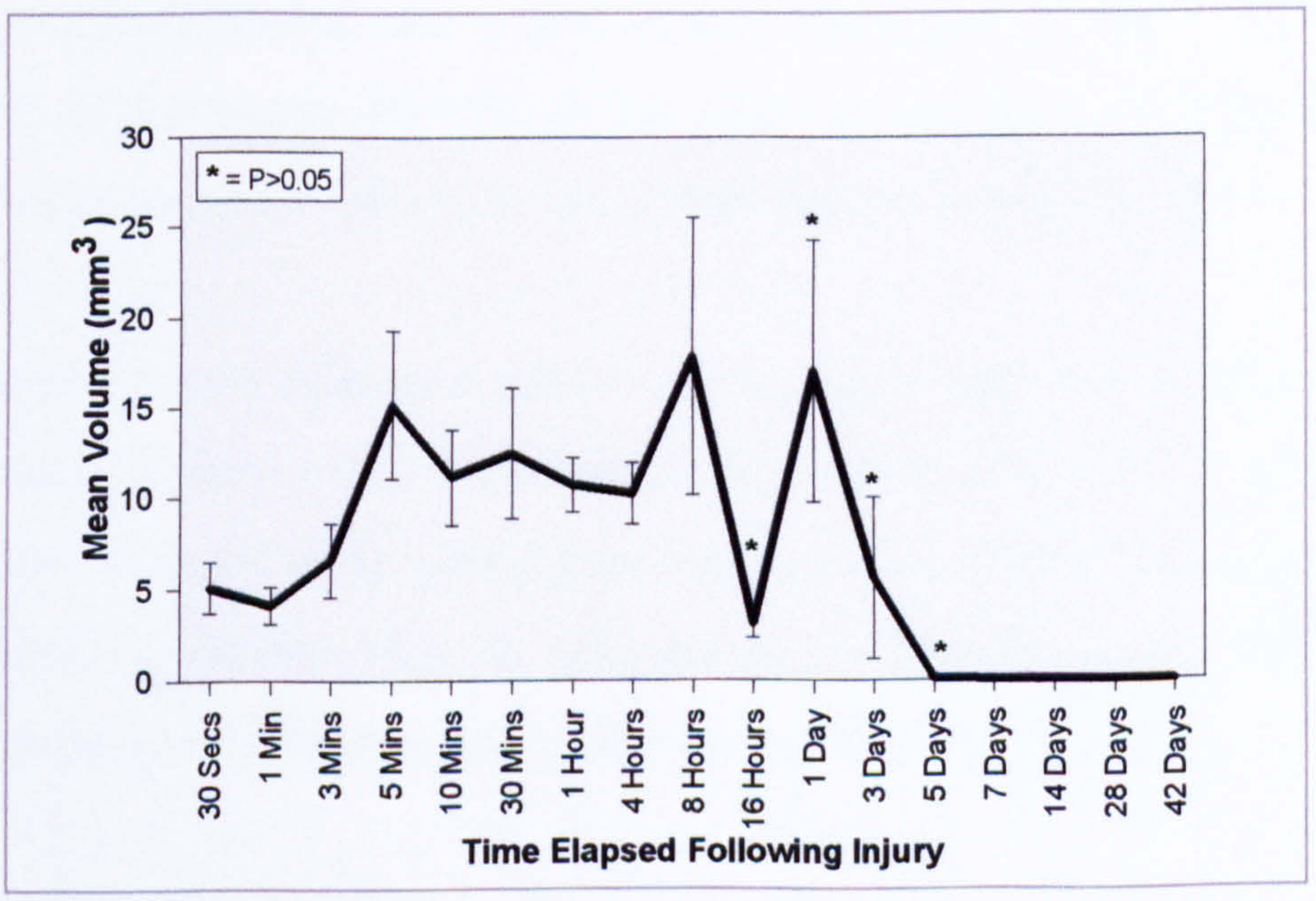
Mean uterine horn haemorrhage was seen at the first sample time point of 30 seconds and remained until 3 days (Graph 3.8; Appendix III Table 3.2). Although there were many fluctuations during this time, the general trend was similar volumes from 30 seconds to 3 minutes, followed by an increase at 5 minutes, with levels remaining elevated up to 1 day, after which a sharp decrease was seen. Significant changes ($P>0.05$) were seen between 8 and 16 hours, 16 hours and 1 day, 1 and 3 days and 3 and 5 days (Appendix III Table 3.3).



Graph 3.6: Mean peritoneal reaction volumes together with SEMs and statistical differences.



Graph 3.7: Mean peritoneal cell concentration volumes together with SEMs and statistical differences.



Graph 3.8: Mean volumes of uterine horn haemorrhage together with SEMs and statistical differences.

The overall coefficient of error was calculated to be 11.4%.

3.4.2 Discussion

The overall incidence of PSAs in this study was found to be 85% (microscopic) to 90% (macroscopic/*in vivo*). These are calculated from the first time point in which PSAs were present in the majority of cases, 8 hours and onwards for microscopic and 10 minutes

and onwards for macroscopic observations. These incidences are comparable to the recently reported clinical incidence of 93% for human post operative intraperitoneal adhesions (Menzies and Ellis, 1990; Duron *et al.*, 1993).

The injury used in this study was more than severe enough to have the potential to induce PSAs in all cases. It is hypothesised that all cases formed primary or fibrinous PSAs in response to the abrasion injury but subsequently some of these fibrinous PSAs underwent lysis, decreasing the incidence of PSAs for the whole of this study. Support for this comes from both observations made by surgeons and this study. Reports that PSAs formed during the early post-operative period spontaneously lyse are prevalent in the surgical community, with a similar situation seen during this study: 100% of cases from 8 hours to 1 day possessed PSAs and it was not until after this time that incidence decreased at some sample time points. Hence fibrinolysis of initial, fibrinous PSAs occurred in approximately 10-15% of the cases in this study, with this phenomena probably due to increased fibrinolytic capacity of those individual animals.

Although experimental sites were adhered *in vivo* in as little as 10 minutes following injury, it was not until 1 hour post injury that these fibrinous PSAs were tenacious enough to remain intact whilst until going tissue resection, slicing and processing. Hence the formation of fibrinous PSAs in response to mechanical injury is very rapid and quicker than previous estimates of 3 hours (Thrompke & Siegner, 1955).

From 4 to 16 hours post injury, maximal increases in PSAs volume were seen, hence a period of intense PSA infrastructural modelling occurred at this development stage. This is probably primarily due to laying down of fibrin and it would appear that the deposition of fibrin is a stimulator of further fibrin deposition. In addition to fibrin deposition we also know that during this time period the inflammatory response of wound healing is being established (Milligan & Raftery, 1974) with PMNL and macrophage involvement. These cells are known for their phagocytotic and chemotactic activity, releasing a wide variety of cytokines and growth factors. This cellular and biochemical activity maybe another stimulator for the deposition of further fibrin shown in this study as the increase

in mean PSA volume.

From 16 hours to 7 days there was a trend for mean volume of PSA to increase. Initially this trend is due to further fibrin deposition, however from 4 days onwards it is most probably due to collagen lay down by fibroblasts (Milligan & Raftery, 1974). In addition cellular infiltration and activity and angiogenesis will also exert an effect on PSA volume. Hence from 16 hours onwards PSA remodelling was occurring, with fibrinous PSAs transforming into established fibrous PSAs. From 7 days onwards a trend for mean PSA volume to decrease was seen. It is hypothesised that this is a result of contraction of collagen bundles (Ellis *et al.*, 1965) and to some extent decreasing cellular presence (Milligan & Raftery, 1974).

Considering PSA prevention, any therapeutic intervention should be directed at these first stages of PSA development, ideally at the time of surgery thus preventing PSA modelling. Failing this lysis of PSAs should be concentrated during the period of PSA modelling, that is the first 16 hours following injury. Any attempts at separation or lysis of PSAs after this time will be increasingly difficult and carry a higher risk of stimulating further PSAs by the action of adhesiolysis.

PSAs, by definition, are not isolated entities and the effects of changes in adjacent and underlying tissues on the development of PSAs has been generally neglected by previous studies. Studies by Ellis (1962) and Belzer (1967) have demonstrated a relationship between damage and PSAs, with the results of this study supporting this, with a direct relationship between the volume of the original injury including tissue changes associated with the original injury and the volume of injury tissue generated from those damaged tissues (PSA and tissue generation). According to these results it is possible that minor contusional injury, not readily seen as operative trauma, may have the potential to induce PSAs. This is in support of those authors who suggest that heavy handling of tissues during surgery results in PSA development (Connolly & Smith, 1960).

The time scale for injury tissue generation was similar in both the peritoneal wall and the

uterine horn and it appears that injury tissue is the only volume change during the first few days of wound healing in this model. After an initial relatively slow injury tissue volume changes, there was a period of significant increase in the amount of tissue generated. For the peritoneum, this time was from day 1 to 14 post injury, after which time tissue generation decreased, whereas for the uterine horn, the increase in volume occurred from 3 to 5 days, and then decreased. This pattern showed a delay in the beginning of the most active phase of tissue generation, which was also seen with PSA development, although this was much shorter, up to 4 hours. This may show that access for PSAs to angiogenesis and other cellular tissue generation stimulators, from both injured tissues, constitutes more efficient tissue growth and therefore faster generation of PSAs when injury tissue connects peritoneal wall to uterine horn.

The decrease in tissue generation observed in both peritoneum and uterine horn following the above mentioned peak in tissue generation activity could be due to: (i) fibrinolysis, but is more probably due to (ii) tissue generation from the injured surfaces becoming part of the PSA, (iii) phagocytosis of tissue generation, and (iv) contraction of collagen bundles within the tissue.

The uterine horn produced less tissue generation at each of the time points investigated which has been a common feature of all previous studies in this laboratory. This is most probably due to the uterine horn sustaining a lower extent of underlying damage compared to the peritoneum during injuring. It is clear from the results of this and previous studies (Ellis, 1962; Belzer, 1967) that there is a relationship between the amount of damage suffered and volume of fibrotic tissue generated. However it is also possible that the peritoneum is capable of greater injury-induced reaction, compared to the uterine horn, in response to this type of injury. Support for this can be found in the fact that at 30 seconds following injury, oedema was detectable at the peritoneal injury site but it was not until 1 hour following injury that any external reaction was noted in the uterine horn.

Although we have considered our classification of tissue generation to be PSA-like

material which is simply not adhered, there may be other differences. This may be supported by the different time scales in which they formed, and the significant decreases in the mean volume of tissue generation present for both the peritoneum and uterine horn, although no significant decrease was seen for PSA volume. A reason for the latter could be simple physical parameters if fibrinolysis is involved, with tissue generation could be more susceptible to attack due to the larger exposed surface area compared to PSA. Equally it is possible that tissue generation only supplied with vascular and cellular support from one injury site may take place at a significantly reduced rate compared to tissue supplied from both injury sites as with PSAs.

For both peritoneum and uterine horn, similar changes were seen with regard to mean volume of damage: constant volumes up to 3 days representing the volume of tissue removed during the injury process, followed by a significant increase and then decrease. This follows the normal progression of wound healing. The abrasion injury process would not only have removed the serosa and some underlying tissue, but would also have damaged underlying and adjacent tissue. This in turn would lead to associated hypoxia and ischaemia and subsequently local tissue necrosis. For proper wound healing to occur, necrotic tissue needs to be removed and all debris cleared from the injury site by phagocytosis. It is this tissue clearance which results in increased damage volume. However once cleared tissue repair occurred and by 42 days, the mean volume of damage for both uterine horn and peritoneal wall was reduced to very minimal levels indicating a successful wound healing process to compensate for damaged/lost tissue.

Clearance of necrotic tissue by phagocytotic cells is highlighted by the correlation between peritoneal cell concentration and peritoneal damage volumes. Following the peak in peritoneal cell concentration at 3 days post injury there was a peak in peritoneal damage at the next time point of 5 days. This suggests that between 3 and 5 days post injury there was very active phagocytic response which was highly effective at damaged tissue removal. Cellular concentrations observed additionally follows the pattern of cellular activity during PSA development. Milligan and Raftery (1974) reported intense cellular infiltration into damage tissue between 1 and 5 days after tissue injury which is

precisely what was seen in this study, with a peak in cellular concentration occurring at 3 days.

Peritoneal cellular concentration results also correspond with that for peritoneal reaction. If we assume that a significant portion of the reaction which we see microscopically is due to biochemical mediators, controlling inflammation and cell infiltration, then the peak seen in this biochemical reaction corresponds with that seen with cellular concentration. Our results indicate that chemotactic activity is occurring within adhered tissues.

Reaction within the peritoneal wall seen at 42 days following injury was still relatively high, especially compared to that seen at the initiation of this study. Wound healing can take several months to be completed so this reaction was not surprising, however levels observed were higher than expected. This could be due to peak levels of reaction being so elevated that a significant amount of time is needed for reaction to subside. In this case, PSA formation must induce a large and sustained reactive tissue response.

Although microscopically we could not distinguish an area of reaction on the uterine horn at the magnification used, haemorrhage was evident up to 3 days following injury. Due to the highly vascular nature of the uterine horn it is not surprising that this type of injury would induce a haemorrhagic reaction. The haemostatic state of the uterine horn was completely restored by 5 days following injury.

Conclusions

i. *In vivo* PSAs were present from 10 minutes following injury, although these initial linkages are relatively weak and tenuous.

ii. Tenacious PSAs were present from 1 hour post injury.

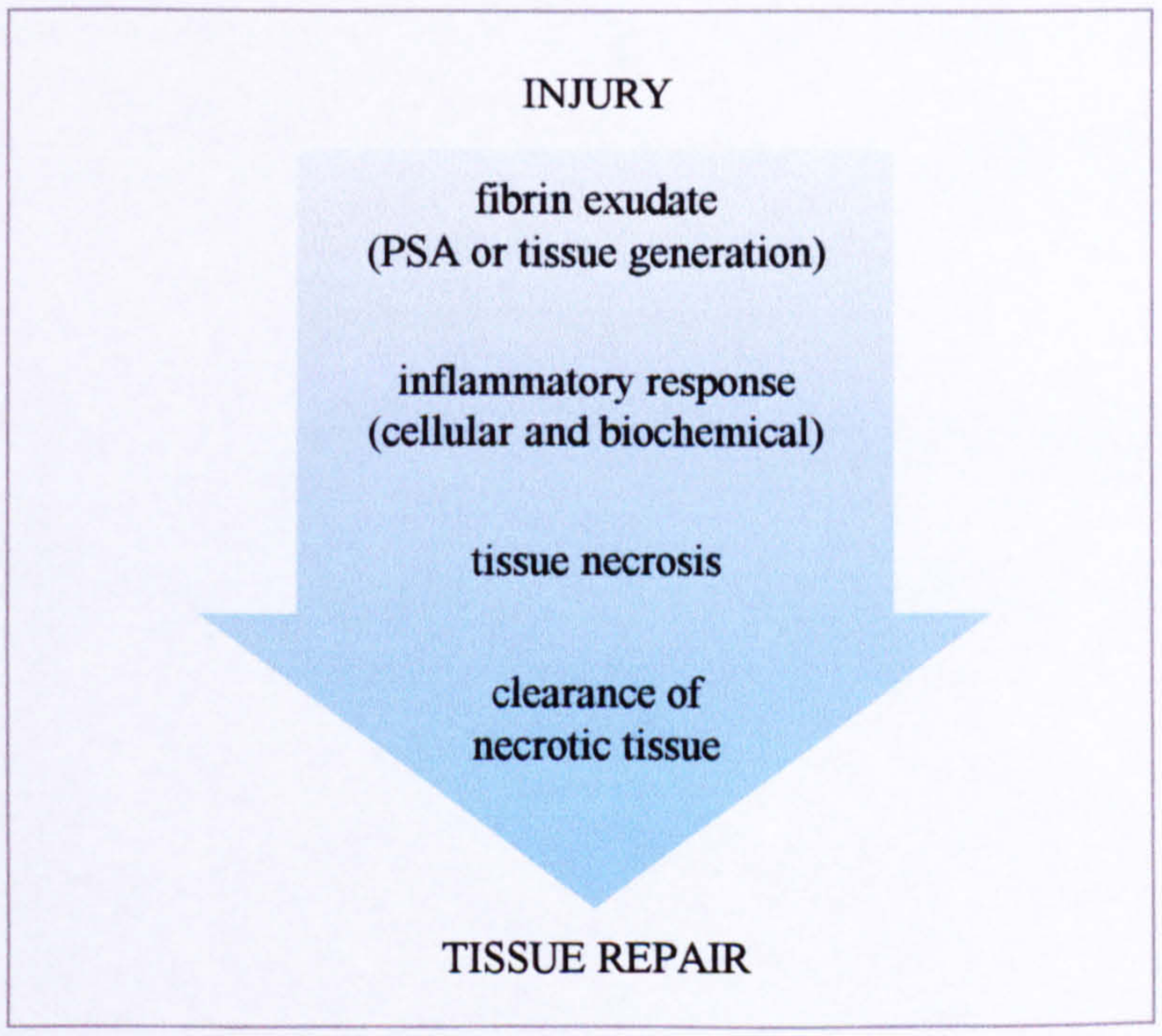
iii. PSA development can be classified into two distinct stages:

- PSA modelling - occurring during the first 16 hours, in which maximum rate of PSA construction transpires.
- PSA remodelling - from 16 hours onwards, in which primary fibrinous PSAs mature into established fibrous PSAs.

iv. An estimated 10 to 15% of fibrinous PSAs were broken down during the course of this study.

v. There was a direct relationship between volume of damage (initial and subsequent) and the volume of injury tissue generated.

vi. PSA development follows the normal pattern of wound healing:



vii. PSA prevention should ideally be initiated immediately post injury to prevent PSA modelling or alternatively during PSA modelling.

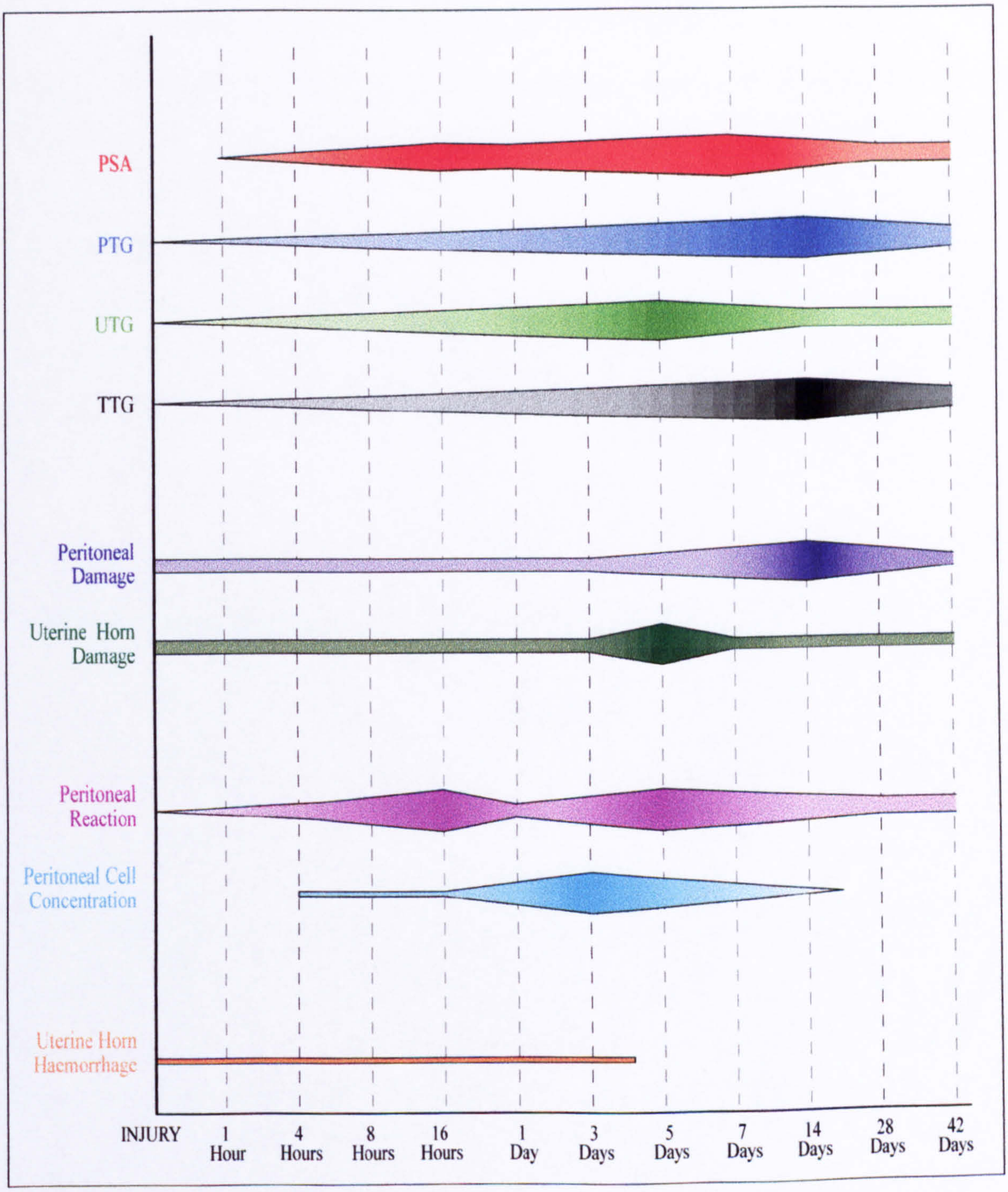


Figure 3.8: Summary of physiological changes occurring during PSA development.

3.5 Volumetric Analysis of Fibrin and Collagen

Fibrin and collagen are the main structural elements present in fibrinous and fibrous PSAs respectively. Milligan & Raftery (1974) demonstrated that up to 3 days post injury PSAs were constructed of a fibrin matrix, with a few collagen fibres first seen at 3 days. After 3 days this equilibrium was reversed with collagen being the predominant structural protein and fibrin nearly totally removed. By 5 days collagen formation was advanced with collagen bundles now present and further maturated seen from thereon. Hence fibrinous PSAs are seen up to and/including 3 days, after which they are transformed into fibrous PSAs.

The aims of this study were to:

i. substantiate the fibrinous and fibrous nature of PSA described previously by quantitatively investigating volumes of fibrin and collagen in developing, experimentally-induced PSAs and tissue generation
ii. compare adhered and non adhered cases within the control group with regard to fibrin and collagen composition and determine structural differences.

Odd numbered slices of all experimental sites were processed to paraffin wax with 5µm sections cut from each block. Sections were stained with PTAH for fibrin detection and/or Van Gieson for collagen detection and analysed using Cavalieri's Principal for determining absolute volumes of fibrin and collagen (refer to Chapter 2).

3.5.1 Results

During the first 16 hours following injury, fibrin only was present in injury tissue (PSAs and tissue generation) and was predominant for the first 3 days (Graph 3.9; Appendix III Table 3.4; Figure 3.9). From 1 to 28 days percentage of fibrin present decreased, with significant decreases between 1 and 3 days ($P>0.01$), 3 and 5 days ($P>0.0001$) and 14 and 28 days ($P>0.05$) (Appendix III Table 3.5).

Collagen was first evident at 1 day following injury, although only accounting for 0.07%

(Graph 3.9; Appendix III Table 3.4). However, from this time point to 28 days mean percentage of collagen increased, with significant increases between 1 and 3 days ($P>0.01$), 3 and 5 days ($P>0.0001$) and 14 and 28 days ($P>0.05$) (Appendix III Table 3.5). By 5 days collagen was the predominant structural protein present, with this trend maintained throughout the remainder of this study (Figure 3.10).

Volume of fibrin increased from 1 to 8 hours, with a significant increase ($P>0.01$) between 1 and 4 hours and volumes remaining similar up to 1 day post injury (Graph 3.10; Appendix III Tables 3.4 and 3.5). There was then a rapid and significant increase ($P>0.01$) between 1 and 3 days, at which time point volume of fibrin peaked. A dramatic decrease occurred from 3 to 5 days, followed by a steady decline with a significant decrease ($P>0.05$) from 14 to 28 days, at which time fibrin was absent.

At 1 day collagen was present in very small volumes and demonstrated a minor but significant increase ($P>0.01$) in volume from 1 to 3 days (Graph 3.10; Appendix III Tables 3.4 and 3.5). From 3 to 14 days collagen volume increased rapidly with significant increases from 1 to 3 days ($P>0.01$) and 3 to 5 days ($P>0.001$). Maximum collagen volume was reached at 14 days and a significant decrease ($P>0.05$) was seen from 14 to 28 days, after which little change was seen.

Figure 3.9: Fibrinous PSA
(3 days post injury) (M_o x4) (stained with PTAH)

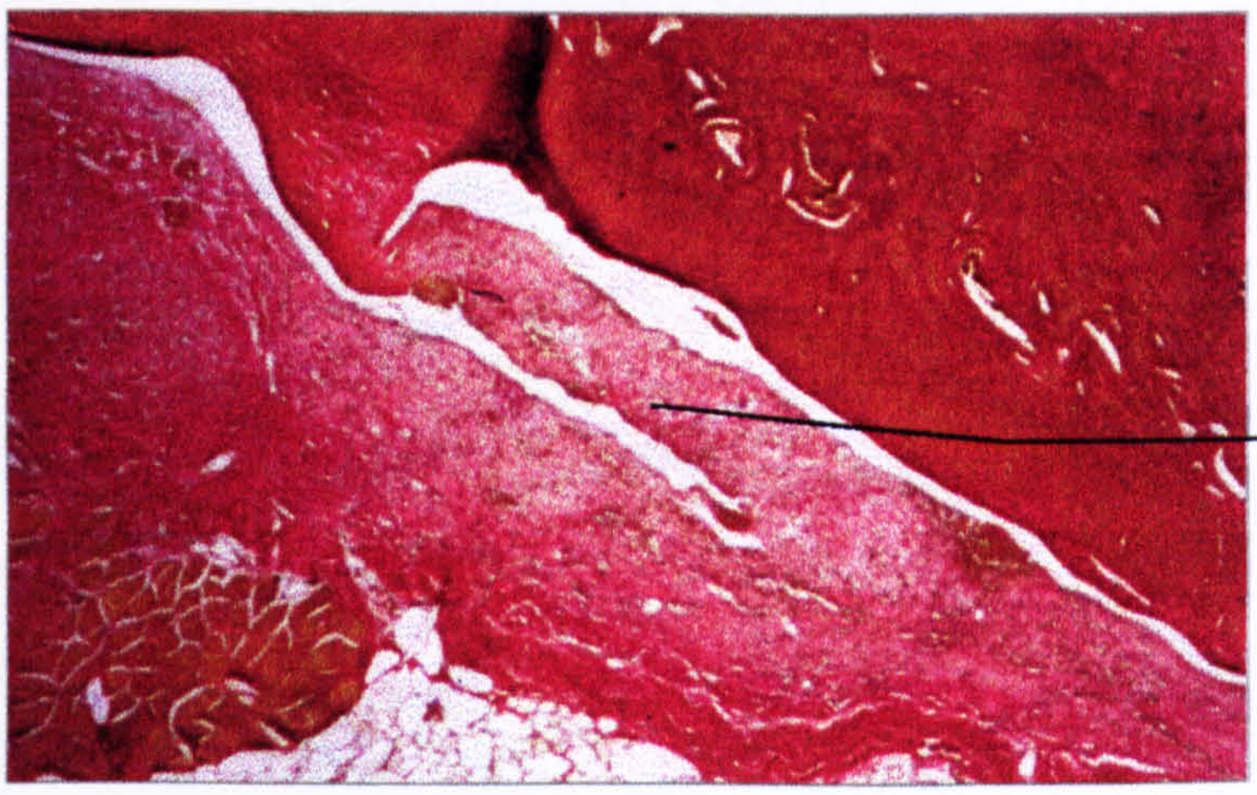
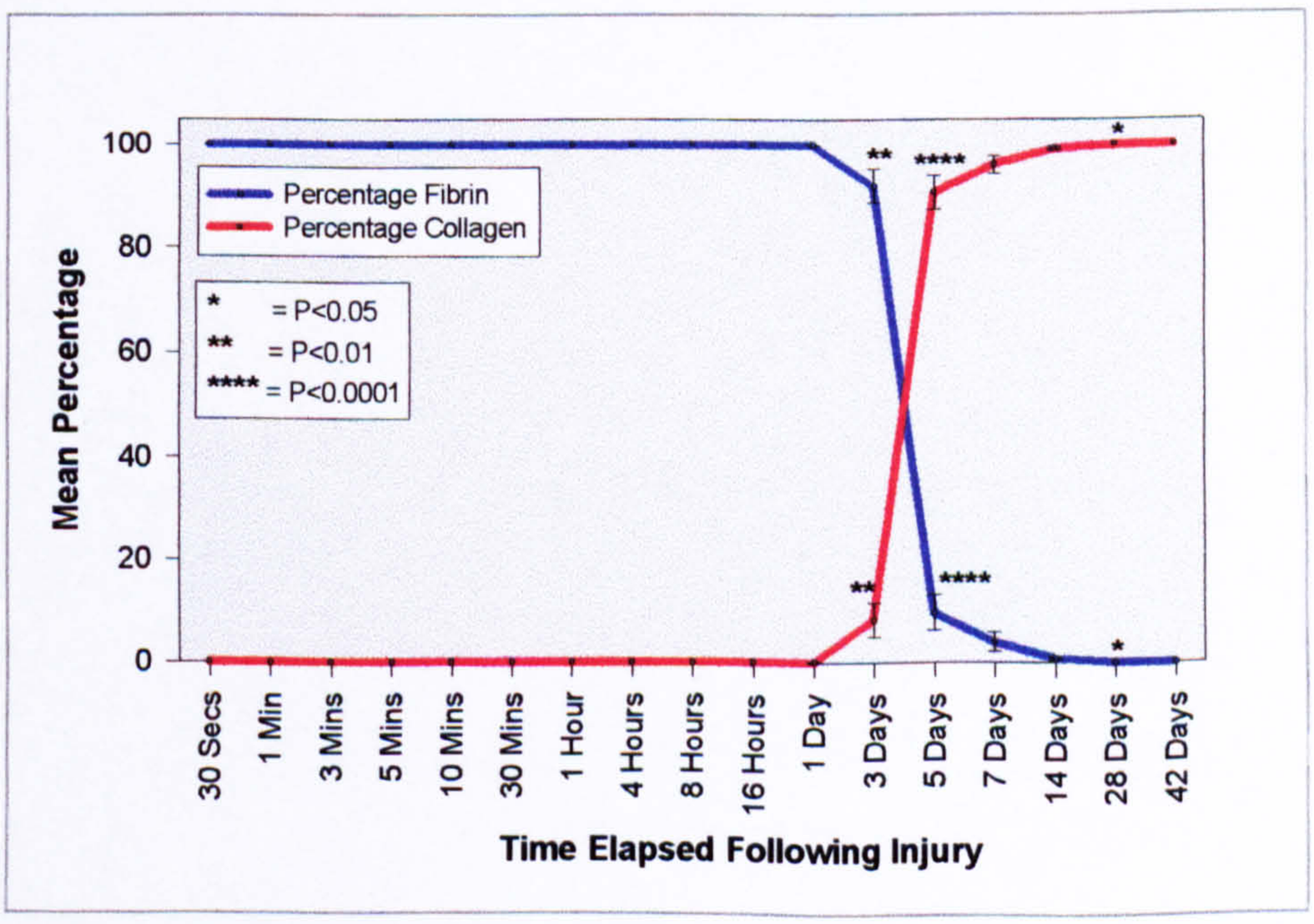
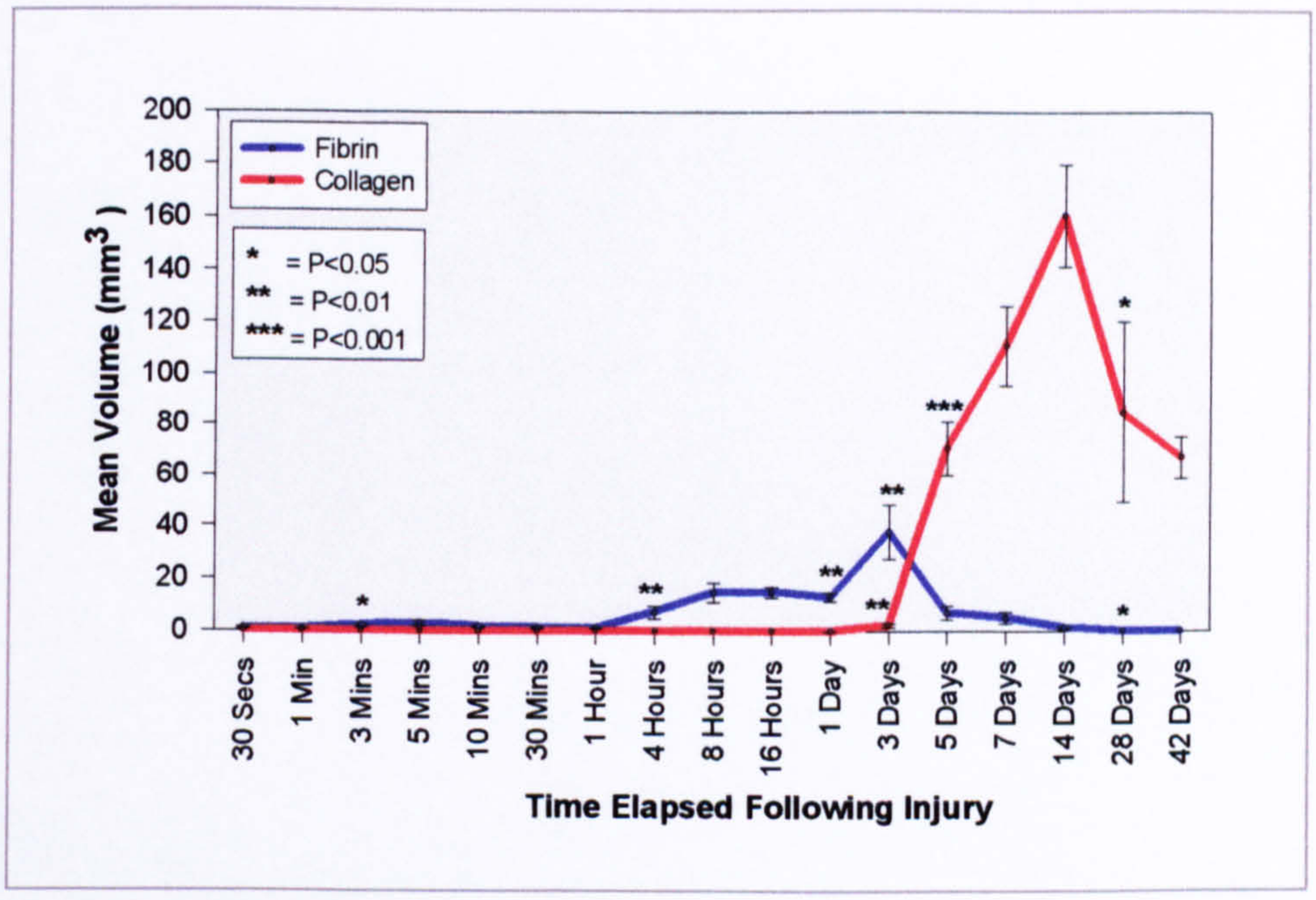


Figure 3.10: Fibrous PSA
(14 days post injury) (M_o x4) (stained with Van Gieson)

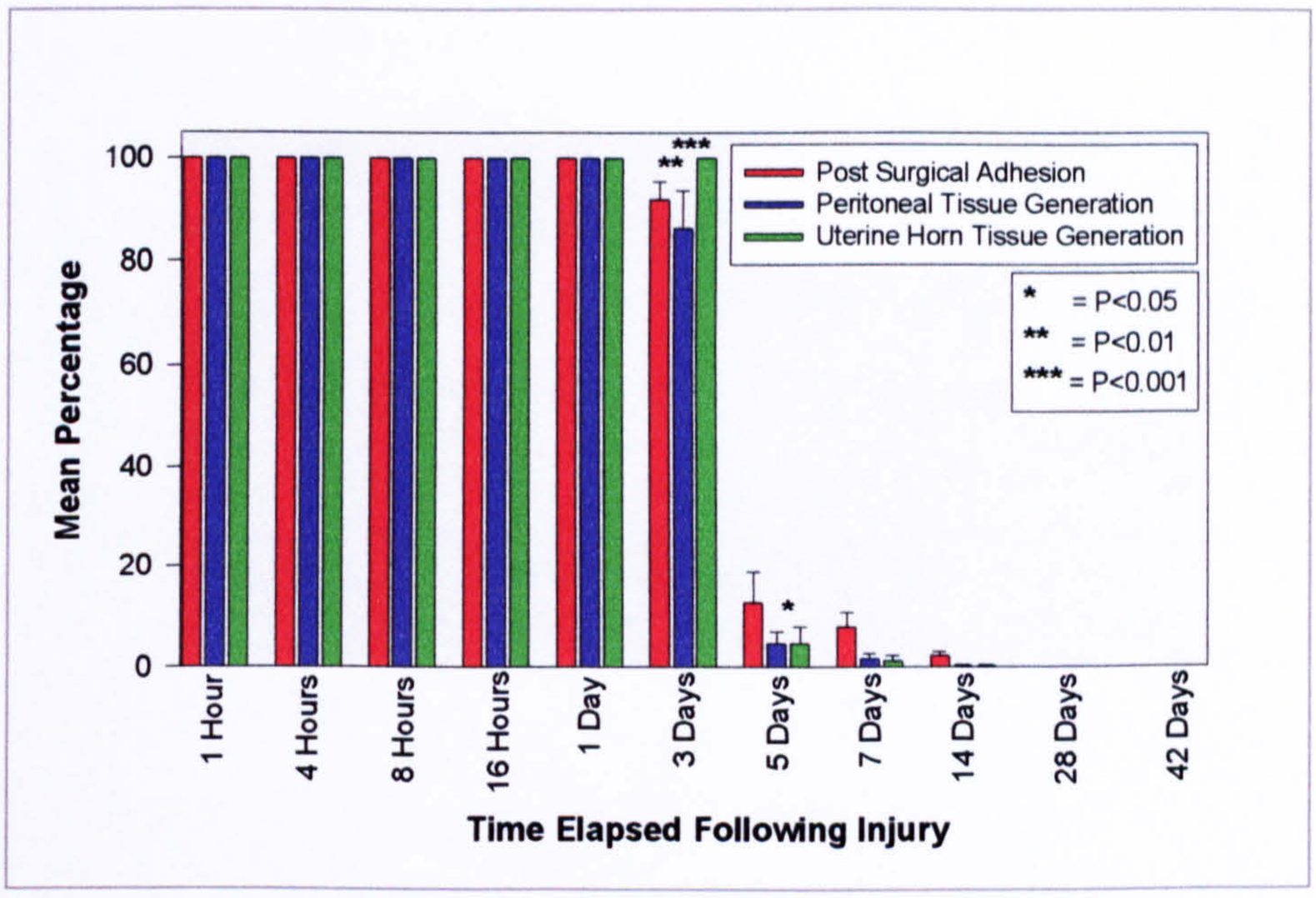


Graph 3.9: Mean fibrin and collagen percentages
together with SEMs and statistical differences.

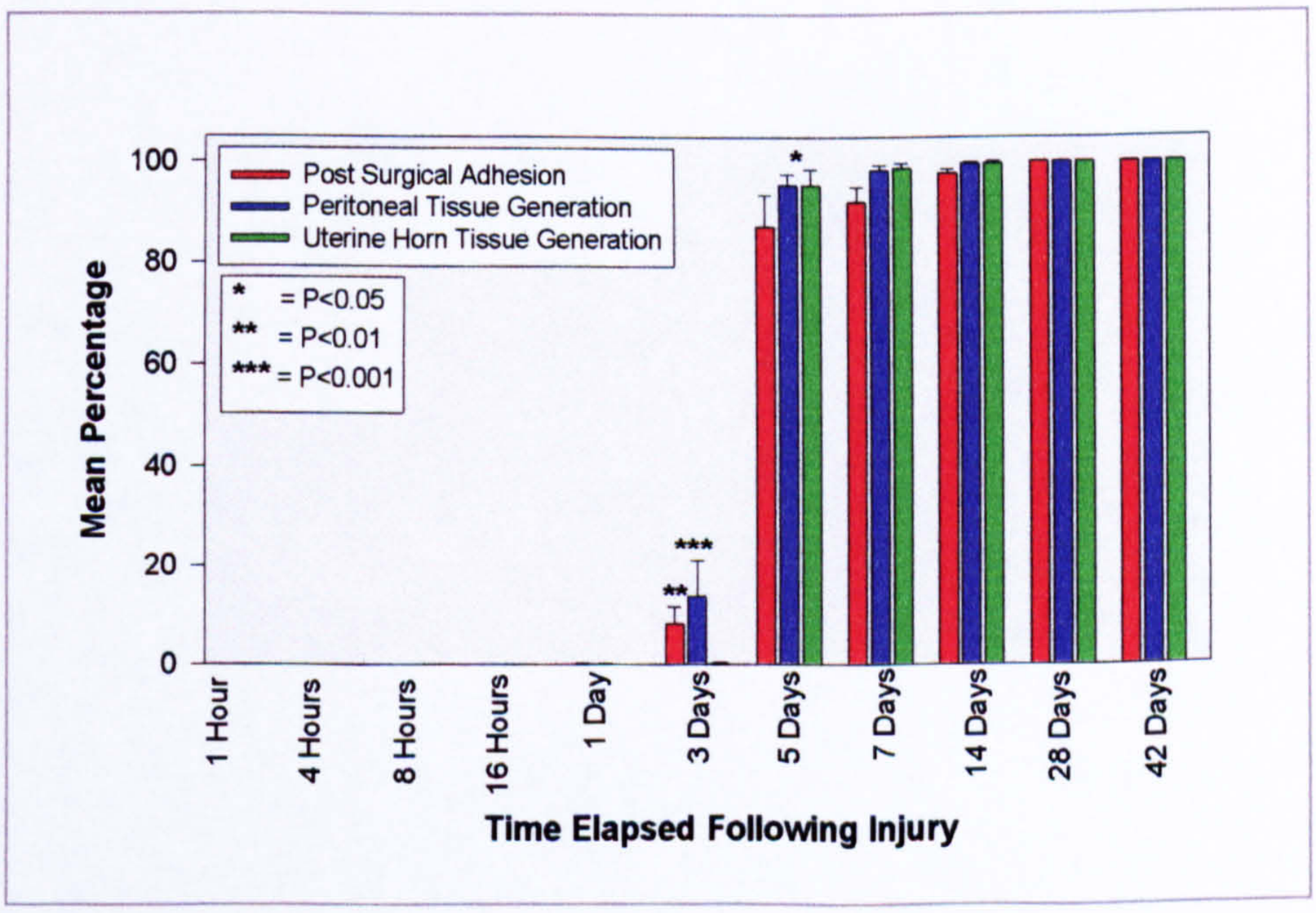


Graph 3.10: Mean fibrin and collagen volumes
together with SEMs and statistical differences.

Comparison of tissue zones demonstrated significant differences between tissue zones at two sample time points following injury (Graphs 3.11 and 3.12; Appendix III Table 3.6). At 3 days, significant differences were demonstrated between UTG and PSA ($P<0.01$) and UTG and PTG ($P<0.001$), with UTG having greater mean percentage of fibrin/lower mean percentage of collagen. At 5 days post injury, significant differences were seen between PSA and PTG ($P<0.05$) and PSA and UTG ($P<0.05$), with PSA demonstrating greater mean percentage of fibrin/lower mean percentage of collagen.



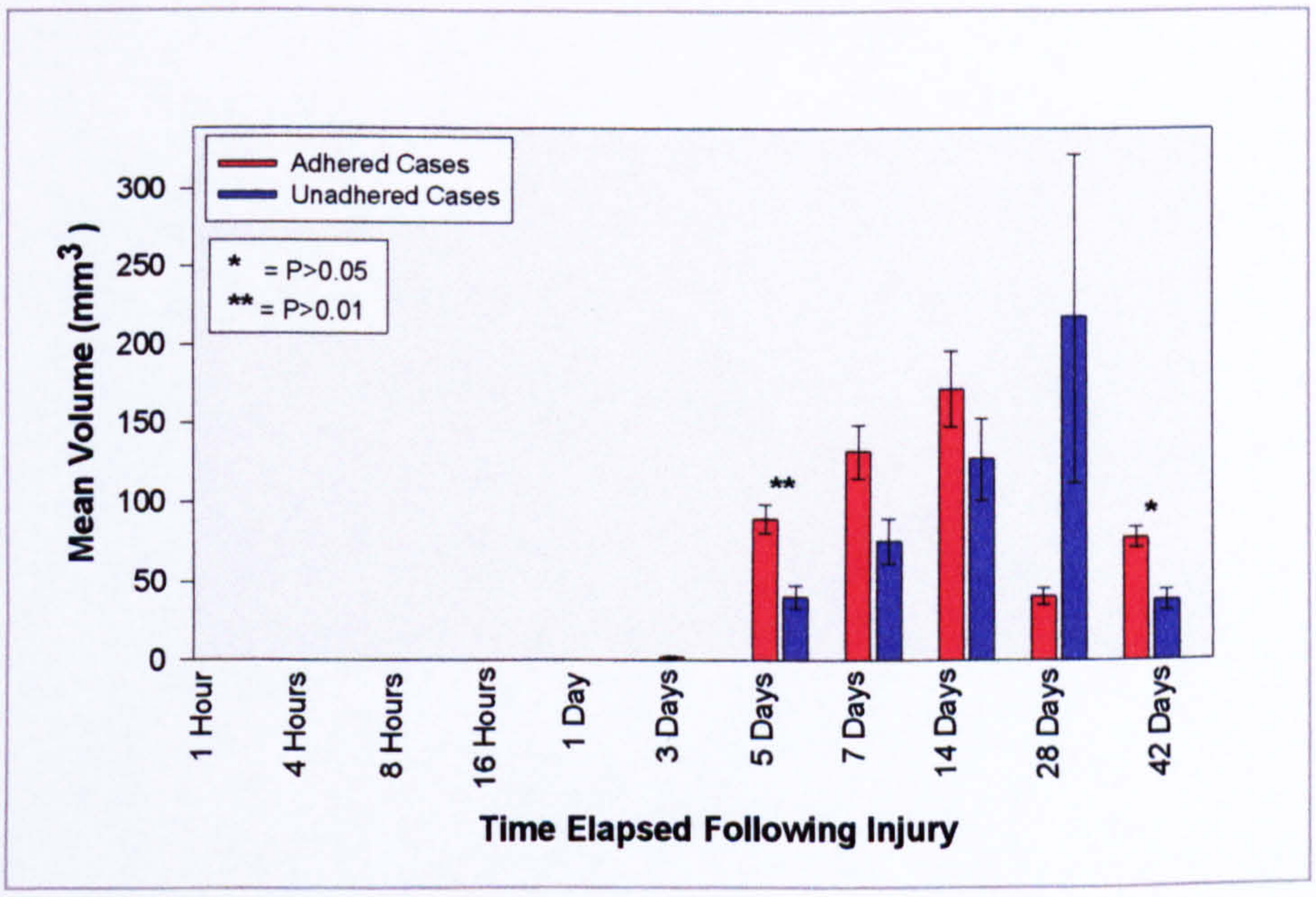
Graph 3.11: Mean fibrin percentages within PSA, PTG and UTG zones together with SEMs and statistical differences.



Graph 3.12: Mean collagen percentages within PSA, PTG and UTG zones together with SEMs and statistical differences.

Analysis of adhered and non-adhered cases revealed a trend for non-adhered cases to have lower mean fibrin volumes at 5 and 7 day time points compared to adhered cases. However significant differences ($P<0.05$) were only seen for mean total volume of collagen at 5 and 42 days post injury, with adhered cases demonstrating greater mean volumes compared to non-adhered cases (Graph 3.13; Appendix III Table 3.7).

The mean coefficient of error for this study was 7.1%.



Graph 3.13: Mean collagen volumes for adhered and non-adhered cases together with SEMs and statistical differences.

3.5.2 Discussion

Fibrin was the predominant structural protein present from 1 hour to 3 days post injury after which it significantly decreased ($P<0.001$), with collagen dominant from 5 days to the end of the study. This correlates with the previous study by Milligan and Raftery (1974) which stated that fibrin was present until 4 days post injury with all remains removed thereafter, and collagen production began at 3 days. In addition data fits with Jackson (1958) who stated that transient PSAs are absorbed by fibrinolysis within 72 hours following injury, hence, during this time, are fibrinous in nature.

PSAs and peritoneal and uterine horn tissue generation are fibrinous up to/including 3 days post injury, after which there is a rapid conversion, with fibrous PSAs seen thereafter. This information is very important with regard to PSA prevention. Fibrinous PSAs have the potent to undergo lysis with minimal tissue injury or chance of stimulating further PSAs, these are therefore the types of PSAs which preventative agents should be targeted against. Lysis or separation of fibrous PSA will be significantly more traumatic, associated with potential tissue injury and subsequent stimulation of PSA formation.

From 30 seconds to 30 minutes, tissue generated in response to injury consisted of blood cell clots on the surface of the peritoneal wall and uterine horn. It was not until 1 hour post injury that fibrin strands were observed together with blood cell clots and this corresponds with the first presence of tenacious PSAs (refer to Section 3.4). Little change was seen at 4 hours, however by 8 hours, fibrin strands had increased and they had formed meshworks. This increased structural strength corresponds with 100% *ex vivo* PSA presence (refer to Section 3.4) and highlights the importance of a fibrin framework for the development of PSAs. Cell involvement was seen together with fibrin up to 3 days and then was less predominant, corresponding with the domination of collagen at 5 days and onwards. Collagen production was first seen at 1 day post injury therefore between 16 hours and 1 day fibroblasts had entered injury tissue and begun production of collagen. Lay down of collagen was rapid between 3 and 14 days when peak collagen volume was seen. After this, collagen volume decreased due primarily to contraction of collagen bundles.

During the course of this study, mean volumes of fibrin and collagen failed to reach the values determined for total tissue generation. Reasons for this are that, initially, some of the tissue identified as tissue generation will be liquid exudate released from the experimental site in response to injury. Further into development, cellular involvement will account for a large proportion of tissue generated, with blood and lymph vessels formed by angiogenesis also adding to tissue generation volume.

During this study all cases sampled from 8 hours to 1 day possessed PSAs whereas

approximately 10-15% of cases sampled from 3 days onwards did not have PSAs present. It is hypothesised that these PSA-free cases did produce fibrinous PSA in response to injury and that these fibrinous PSAs were present up to 1 day post injury after which they were lysed. Hence natural fibrinolysis of fibrinous PSAs occurred between 1 and 3 days following injury which correlates with the time in which fibrin volume was decreasing and collagen production began in earnest. It would appear from the data, that this is the period in which fibrinous PSAs are most susceptible to lysis/division.

Adhered control cases demonstrated a trend for greater mean percentages and volumes of fibrin at 5 and 7 days compared to non-adhered control cases at these sample time points. Since no significant differences were seen for total mean volumes of these tissue zones (Appendix III Tables 3.8 and 3.9) the mean fibrin volumes are true differences. This supports the hypothesis that non-adhered cases have greater fibrinolytic capacities compared to adhered cases. In addition, the physical factor of exposed surface area of fibrin available for fibrinolysis could play a role in this increased amount of fibrin present in adhered compared to non-adhered cases.

These hypotheses are supported by comparison of fibrin and collagen composition in PSAs and tissue generation zones which revealed that fibrin tends to remain in PSAs for a longer time compared to tissue generation zones. In addition there is a trend for fibrin to remain present in UTG longer than PTG. Since these observations are taken from percentage comparisons, peritoneal and uterine horn tissue generation have a trend to have greater collagen composition compared to PSA. This is directly accountable to fibroblasts either being greater in number or a similar number laying down more collagen, with the former theory the most probable due to easier access to tissue generation zones. Analysis of numerical densities and total numbers of cells within tissue zones will clarify this matter.

Conclusions

i. PSAs and tissue generation are primarily composed of fibrin during the first 3 days

following injury and are termed fibrinous.

ii. PSAs and tissue generation are primarily composed of collagen from 5 days onwards and are termed fibrous.

iii. PSA prevention should be aimed at fibrinous PSAs rather than fibrous PSAs.

iv. The composition of injury tissue varies throughout PSA development:

- Between 30 seconds to 30 minutes tissue was composed of blood cell clots produced during haemostasis
- From 1 to 4 hours fibrin strands were present resulting in the formation of tenacious PSAs
- From 8 hours to 3 days a fibrin meshwork was present, resulting in 100% incidence of PSAs
- From 1 day onwards collagen strands were present. These aligned in collagen bundles by 5 days when collagen was predominant leading to the formation of established fibrous PSAs. Contraction of collagen bundles occurred from 14 days resulting in reduced volume.

v. Approximately 10-15% of fibrinous PSAs were lysed during this study between 1 and 3 days. This appears to be a susceptible time for fibrinous PSA lysis.

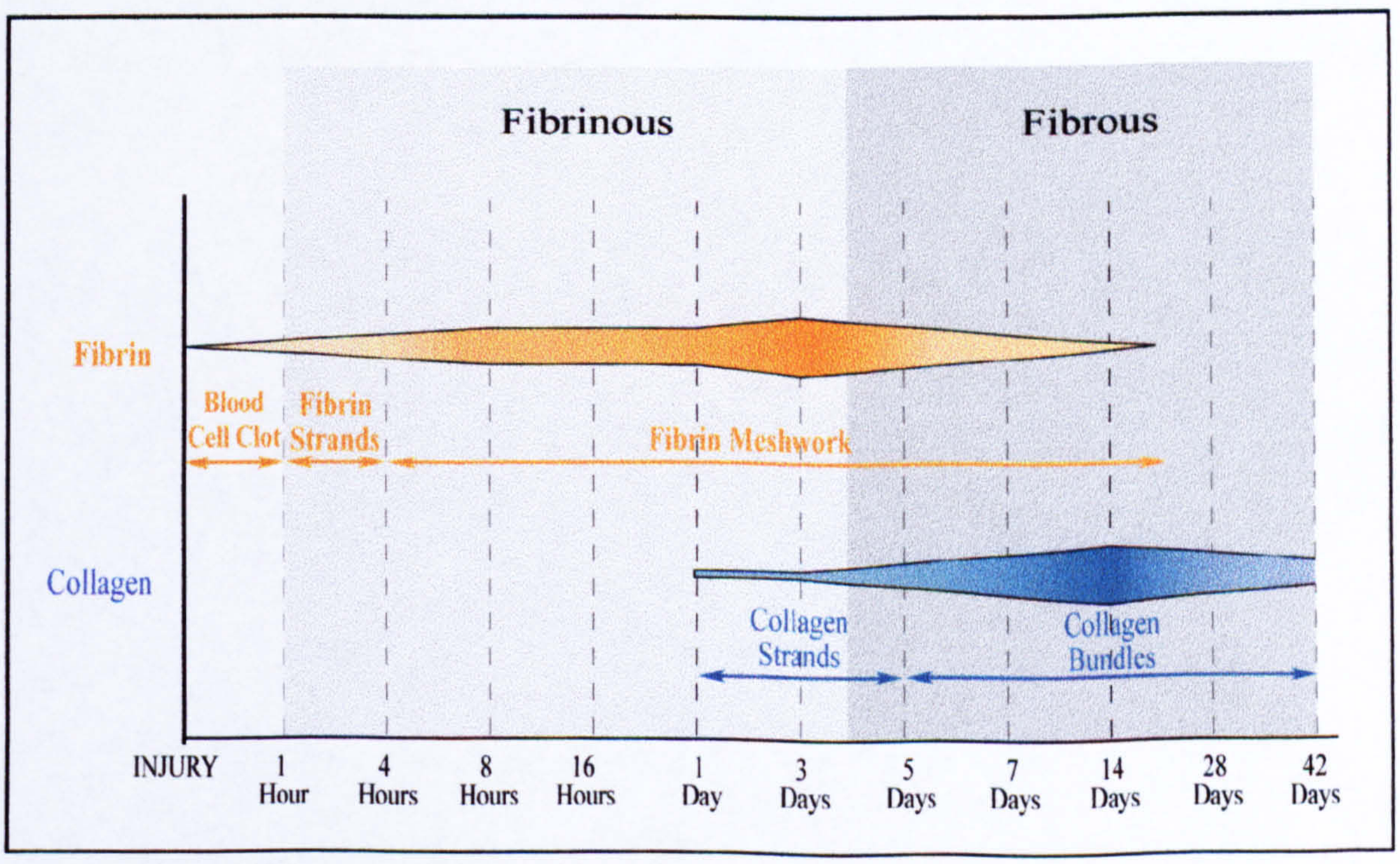


Figure 3.11: Summary of fibrin and collagen changes during PSA development.

3.6 Number Analysis of Cellular Components

Due to the nature of PSAs there is high cellular content and activity throughout their formation and maturation. Milligan & Raftery (1974) demonstrated that PMNLs were the predominant cell type present in PSAs at 1 day following their induction, however macrophages and eosinophils were also present. By 2 days macrophages had become more established, sharing the predominance with PMNLs, with lymphocytes and eosinophils also present. During the next day numbers of PMNLs decreased with macrophages, followed by eosinophils, predominant. Fibroblasts increased in numbers from 4 to 7 days with numbers of macrophages and eosinophils decreasing. By 14 days and onwards cells were relatively sparse, with fibroblasts and occasional macrophages seen (refer to Section 1.1.6.5.2).

Morphological differences exist between human and several mammalian species, with the main difference between rabbit (Appendix III Table 3.16) and human blood cells is that rabbit neutrophils contain granules which stain pink, that is eosinophilic granules, which are absent in human neutrophils. This difference has lead to confusion with cell identification of rabbit blood cells over the years. For the purpose of this study, granular cells which contained eosinophil granules were termed eosinophilic cells and those with only basophil granules termed basophilic cells. In addition monocytes and lymphocytes were counted.

Although Milligan & Raftery (1974) detailed differences in cell presence through PSA development no study has quantified cellular presence. Thus aims of this study were to:

i. quantify the numbers of basophilic cells, eosinophilic cells, macrophages, lymphocytes and fibroblasts present in PSA and peritoneal and tissue generation from 1 hour to 42 days following injury
ii. identify cellular trends influencing PSA development.

Even number slices obtained from uniform random slicing of experimental sites were processed to resin. A section was cut from each resin block at a thickness of 25μm,

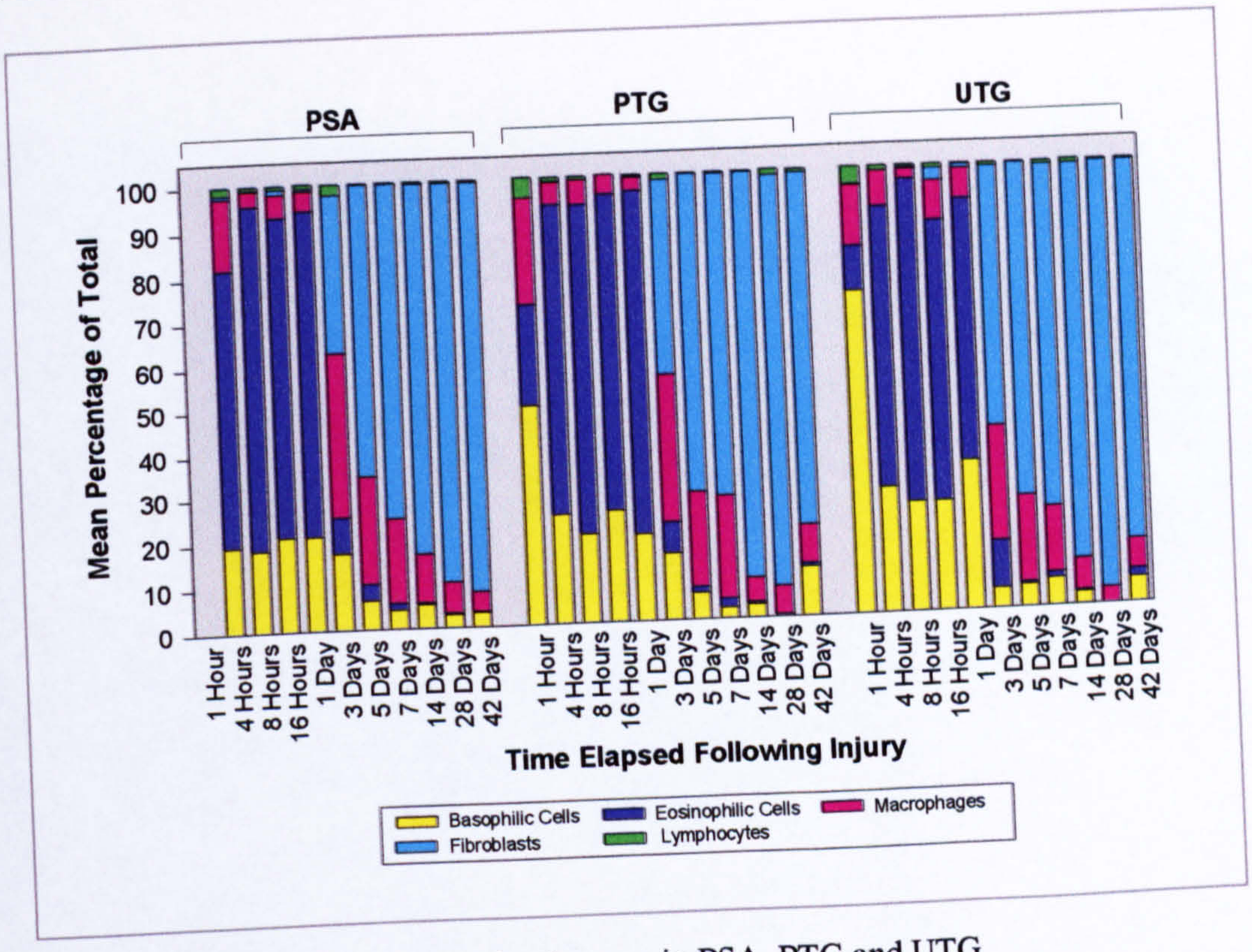
stained with a modified Giemsa stain and analysed using a stereological technique, the optical brick, for determining number density. Total numbers were then calculated from numbers densities and volumes (refer to Chapter 2).

3.6.1 Results

3.6.1.1 Number Densities

3.6.1.1.1 Cell Predominance

Number density predominance of cells investigated varied throughout PSA, PTG and UTG formation and development (Graph 3.14; Appendix III Table 3.10). From 1 to 4 hours basophilic cells were the predominant cell type, followed by eosinophilic cells, macrophage, lymphocytes and fibroblasts respectively. There was a slight change seen between 1 and 4 hours with eosinophilic cells becoming the predominant cell type over basophilic cells. This pattern remained unchanged up to/including 1 day post injury, after which there was a significant shift in cell predominance's. From 3 days (5 days for PSAs) until the end of the study, fibroblasts were the predominant cell types followed by, in order of predominance, macrophages, basophilic cells, eosinophilic cells and lymphocytes. Hence inflammatory cells (eosinophilic cells, basophilic cells and macrophages) were predominant for the first day following injury after which tissue repair cells (fibroblasts and macrophages) were predominant (Figures 3.12 and 3.13).



Graph 3.14: Number density cell predominances in PSA, PTG and UTG.

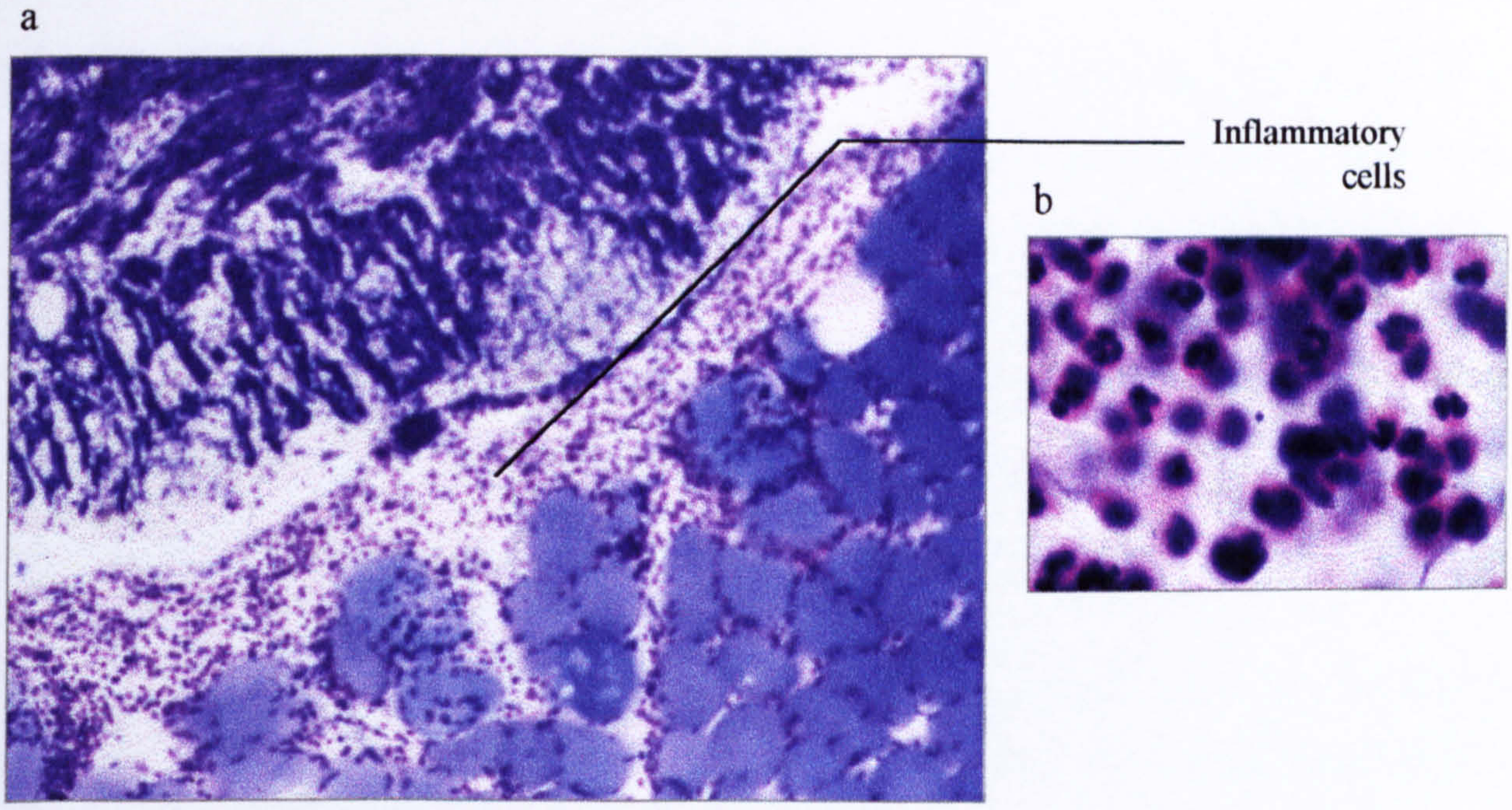


Figure 3.12: Inflammatory cell predominance at 16 hours post injury.
(a= M_o x10; b= M_o x100)

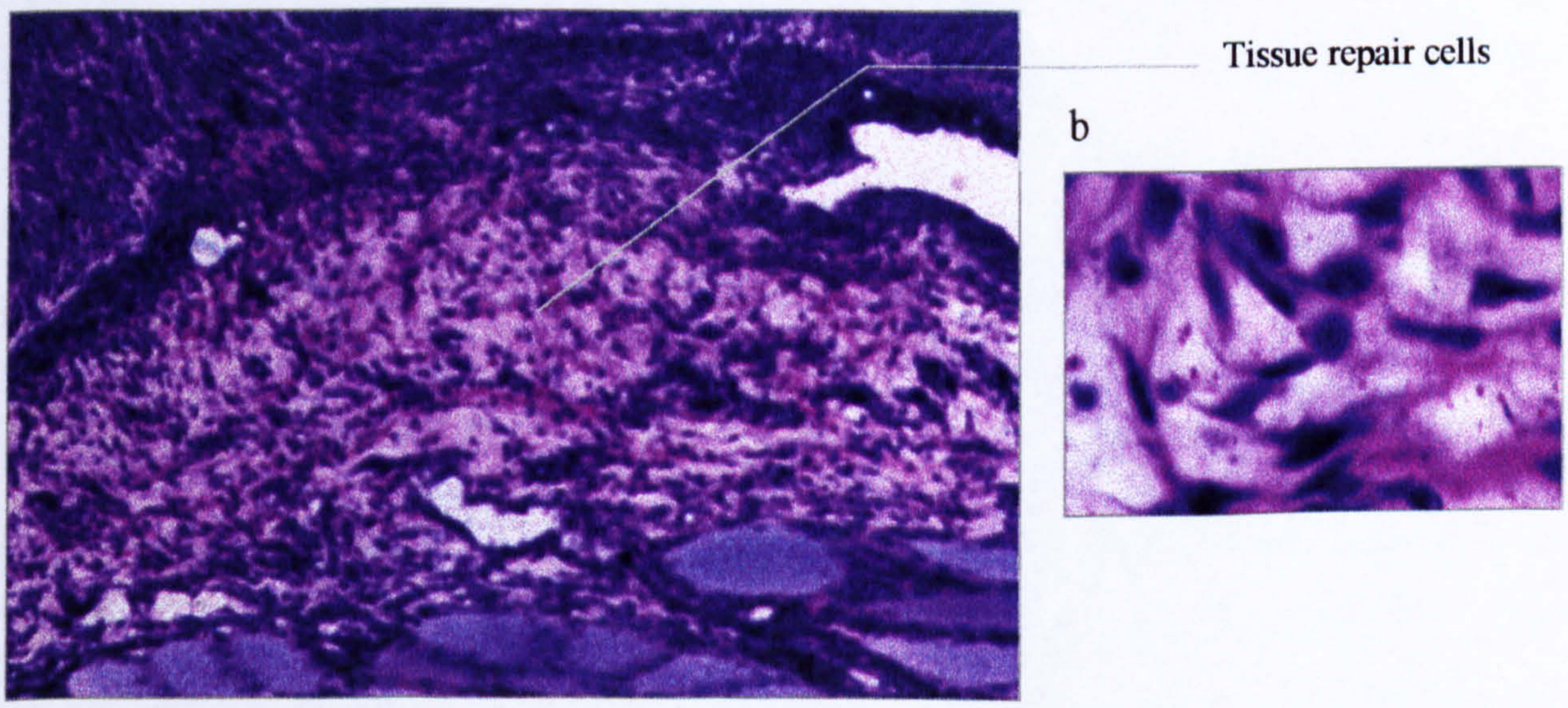


Figure 3.13: Tissue repair cell predominance at 5 days post injury.
(a= M_o x10; b= M_o x100)

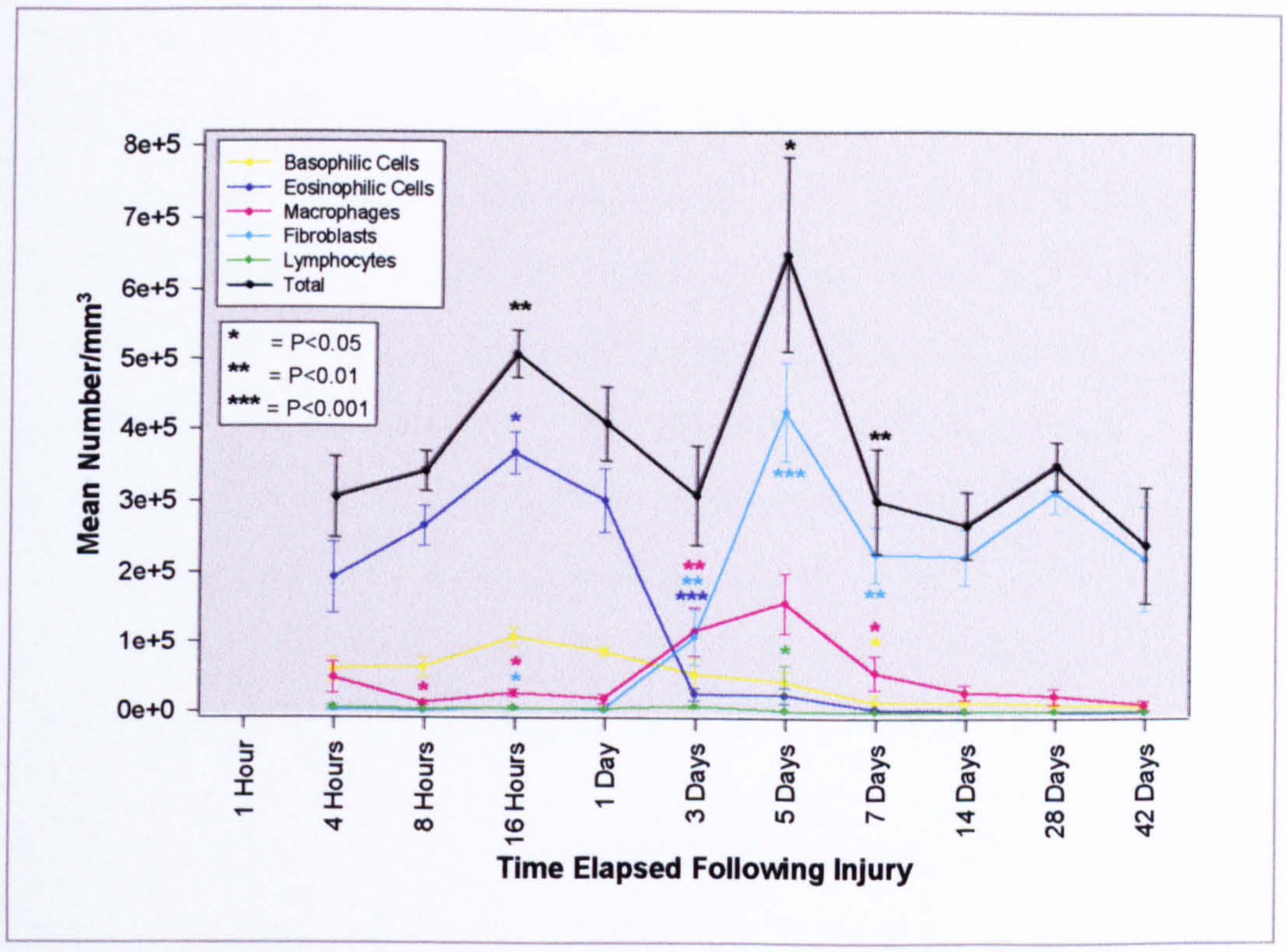
3.6.1.1.2 Post Surgical Adhesions (PSAs)

Number density for all cells analysed (total cells) within PSAs demonstrated an increase from 4 to 16 hours, peaking at 16 hours with a significant increase seen ($P<0.01$) from 8 to 16 hours (Graph 3.15; Appendix III Table 3.10 and 3.11). From 16 hours to 3 days there was a slight trend to decrease followed by a significant increase ($P<0.05$) from 3 to 5 days post injury. This second peak in number density at 5 days was transient with a significant decrease ($P<0.01$) from 5 to 7 days, with a trend for mean total cell density to decrease there after.

Basophilic cell number density within PSAs showed an increase from 4 to 16 hours, peaking at 16 hours (Graph 3.15; Appendix III Table 3.10 and 3.11). Mean cell density then decreased until the end of the study, with a significant decrease ($P<0.05$) seen between 5 and 7 days. Eosinophilic cells demonstrated a similar trend to basophilic cells. From 4 hours onwards mean cell density increased to peak at 16 hours, with this increase significant ($P<0.05$) from 8 to 16 hours. From 16 hours to 42 days eosinophilic cell density decreased with a significant decrease ($P<0.001$) from 1 to 3 days (Graph 3.15; Appendix III Tables 3.10 and 3.11).

Number density of macrophages and fibroblasts followed very similar trends throughout PSA development (Graph 3.15; Appendix III Tables 3.10 and 3.11). From 4 hours to 1 day there was little change, however, from 1 to 5 days there was an increase with number density peaking at 5 days post injury. This increase in mean density was significant for macrophages ($P<0.01$) from 1 to 3 days and for fibroblasts between 1 and 3 days ($P<0.01$) and 3 to 5 days ($P<0.001$). After peaks at 5 days both macrophages and fibroblasts significantly decreased between 5 and 7 days ($P<0.05$ and $P<0.01$ respectively) with macrophages showing a further decrease for the remainder of the study whereas fibroblast density remained relatively constant.

There was little change in number density of lymphocytes throughout PSA development, however, mean density up to 3 days post injury showed a trend to be slightly greater than those after 3 days (Graph 3.15; Appendix III Tables 3.10 and 3.11).



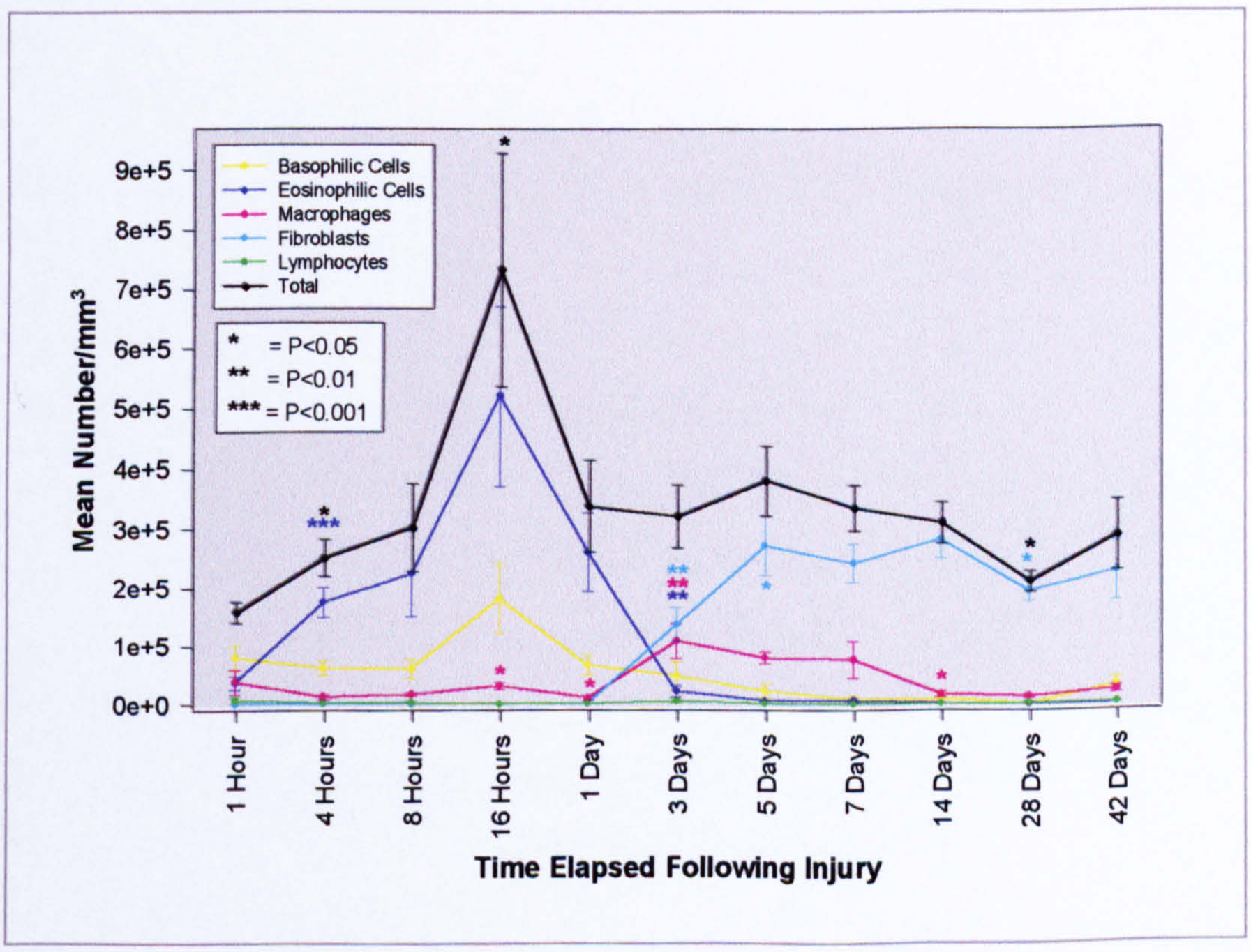
Graph 3.15: Mean cell density throughout PSA development together with SEMs and statistical differences.

3.6.1.1.3 Peritoneal Tissue Generation (PTG)

Number density for total cells within PTG showed an increase from 1 to 16 hours, with significant increases ($P<0.05$) between 1 and 4 hours and 8 to 16 hours (Graph 3.16; Appendix III Tables 3.10 and 3.11). From 16 hours to 1 day there was a sharp decrease in total cell density followed by levels demonstrating a very slight trend to decrease until the completion of this study.

Basophilic cell density within PTG remained unchanged from 1 to 8 hours post injury after which it increased to peak at 16 hours (Graph 3.16; Appendix III Tables 3.10 and 3.11). Following this, density decreased steadily for the remainder of the study. Eosinophilic cells increased from the first sample time point of 1 hour to peak at 16 hours, with a significant increase ($P>0.001$) between 1 and 4 hours. From 16 hours to 3 days there was a sharp decrease in density, being significant from 1 to 3 days ($P<0.001$), with the trend to decrease continued there after (Graph 3.16; Appendix III Tables 3.10 and 3.11).

Macrophage density were relatively constant for the first day of this study, with a significant increase ($P<0.01$) between 1 and 3 days (Graph 3.16; Appendix III Tables 3.10 and 3.11). Mean density plateauxed from 3 to 7 days after which there was a significant decrease ($P<0.05$) from 7 to 14 days, with levels remaining at this minimal value for the rest of the study. The density of fibroblasts within PTG did not demonstrated any change until after 1 day when there were significant increases from 1 to 3 days ($P<0.01$) and 3 to 5 days ($P<0.05$) (Graph 3.16; Appendix III Tables 3.10 and 3.11). From 5 days onwards mean density remained stable with a very slight decrease seen overall. Mean density of lymphocytes throughout PTG formation and development remained constant (Graph 3.15; Appendix III Tables 3.10 and 3.11).



Graph 3.16: Mean cell densities throughout PTG development together with SEMs and statistical differences.

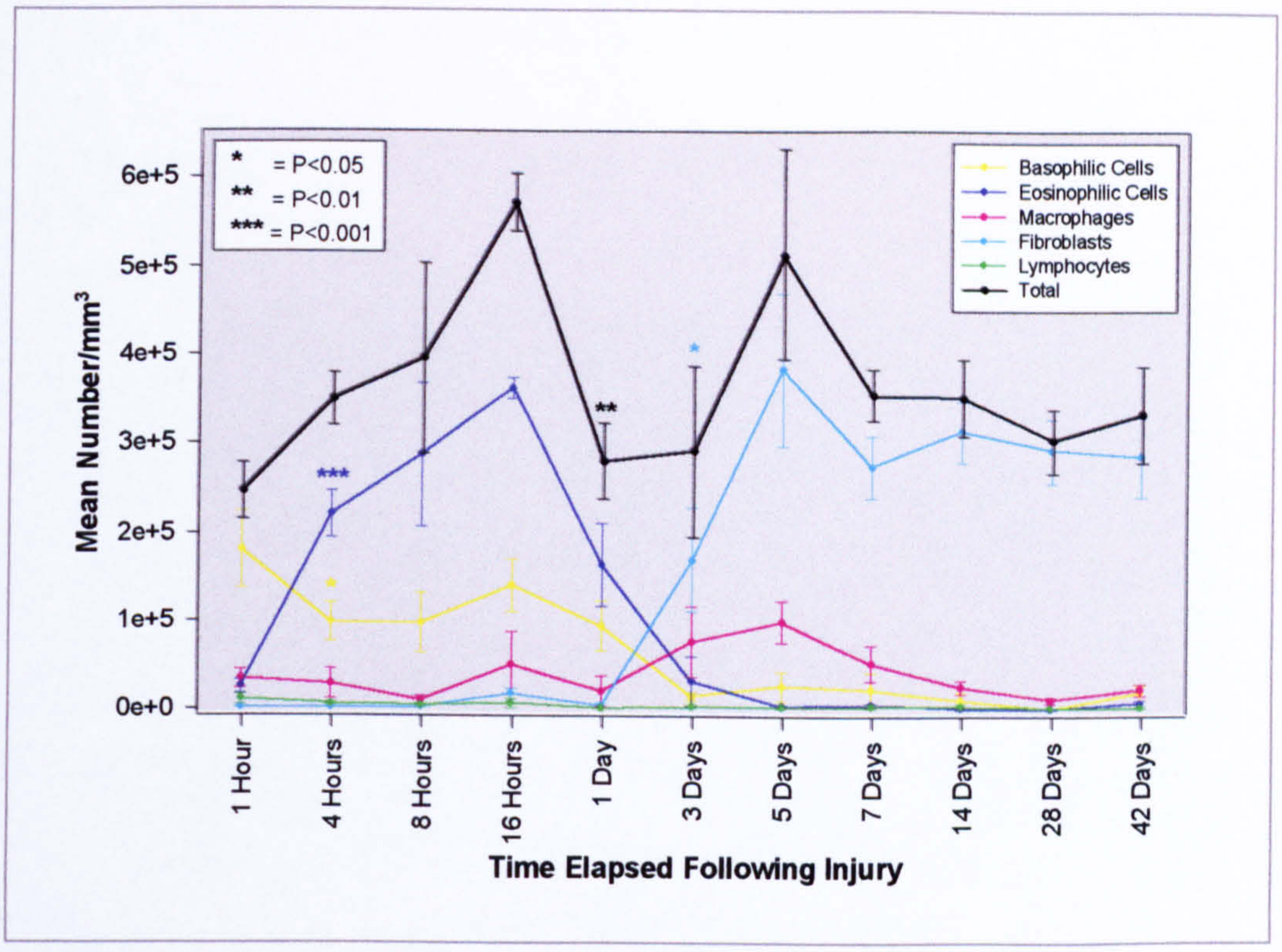
3.6.1.1.4 Uterine horn tissue generation (UTG)

Total cell density within UTG showed an increase from 1 to 16 hours peaking at this time point (Graph 3.17; Appendix III Tables 3.10 and 3.11). From 16 hours to 1 day there was a significant decrease ($P<0.01$) in mean density which remained unchanged at

3 days. There was another increase between 3 and 5 days and a second peak in mean total cell density. From 5 to 7 days there was a decrease in mean density with little change there after.

Basophilic cell density within UTG decreased significantly ($P<0.05$) from 1 to 4 hours post injury (Graph 3.17; Appendix III Tables 3.10 and 3.11). Mean density showed a slight but gradual increase from 4 to 16 hours, with a slight peak at 16 hours. From 16 hours to 3 days, levels decreased and little change was seen throughout the remainder of this study. Eosinophilic cells demonstrated a progressive trend to increase from 1 to 16 hours, with a significant increase ($P<0.001$) in mean cell density between 1 and 4 hours. After peaking at 16 hours post injury, eosinophilic cell density decreased sharply until 5 days, after which density were comparatively stable (Graph 3.17; Appendix III Tables 3.10 and 3.11).

Macrophage density varied little from1 hour to 1 day, although a slight peak was evident at 16 hours. From 1 to 5 days there was a trend to increase, with a peak at 5 days and subsequently numbers decreasing until 42 days post injury (Graph 3.17; Appendix III Tables 3.10 and 3.11). Fibroblasts were at their minimal density from 1 hour to 1 day (Graph 3.17; Appendix III Tables 3.10 and 3.11). From 1 to 5 days there was a rapid increase in cell density, with a significant increase from 1 to 3 days ($P<0.05$). From 5 to 7 days mean density decreased with consistent densities seen thereafter. As with PSA and PTG, lymphocyte density throughout UTG development demonstrated no significant changes (Graph 3.17; Appendix III Tables 3.10 and 3.11).



Graph 3.17: Mean cell densities throughout UTG development together with SEMs and statistical differences.

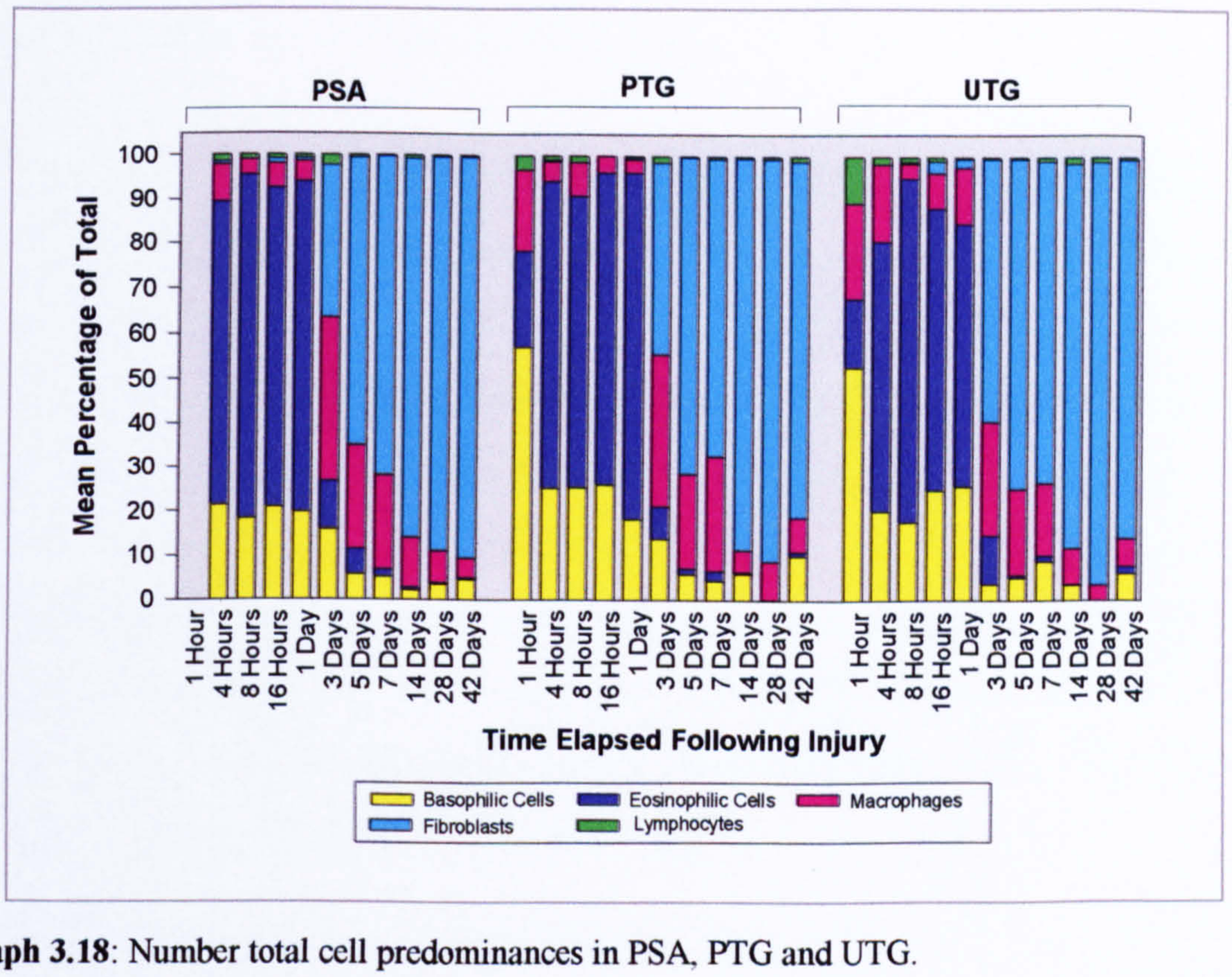
T test analysis of number densities within PSA, PTG and UTG identified significant differences for basophilic cells, fibroblasts and cell totals. Number densities of fibroblasts and totals were significantly greater in PTG compared to PSA at 5 days ($P<0.05$) and 28 days ($P<0.01$) following injury. However number densities were significantly greater ($P<0.05$) in UTG compared to PTG for the following: basophilic and total cells at 1 hours; total cells at 4 hours; fibroblasts at 16 hours; and fibroblasts and totals cells at 28 days following injury. There were no significant differences for number densities between PSA and UTG at any time point (Appendix III Table 3.12).

3.6.1.2 Total Numbers

3.6.1.2.1 Cell Predominance

Total numbers of basophilic cells, eosinophilic cells, macrophages, fibroblasts and lymphocytes within PSA, PTG and UTG for each case were calculated by multiplying number densities by the relevant volumes (determined using Cavalieri's Principal). Due to the determination of total numbers, total cell number predominances were the

same as those for number densities however there were minor differences with regard to cell percentages (Graph 3.18; Appendix III Table 3.13).



Graph 3.18: Number total cell predominances in PSA, PTG and UTG.

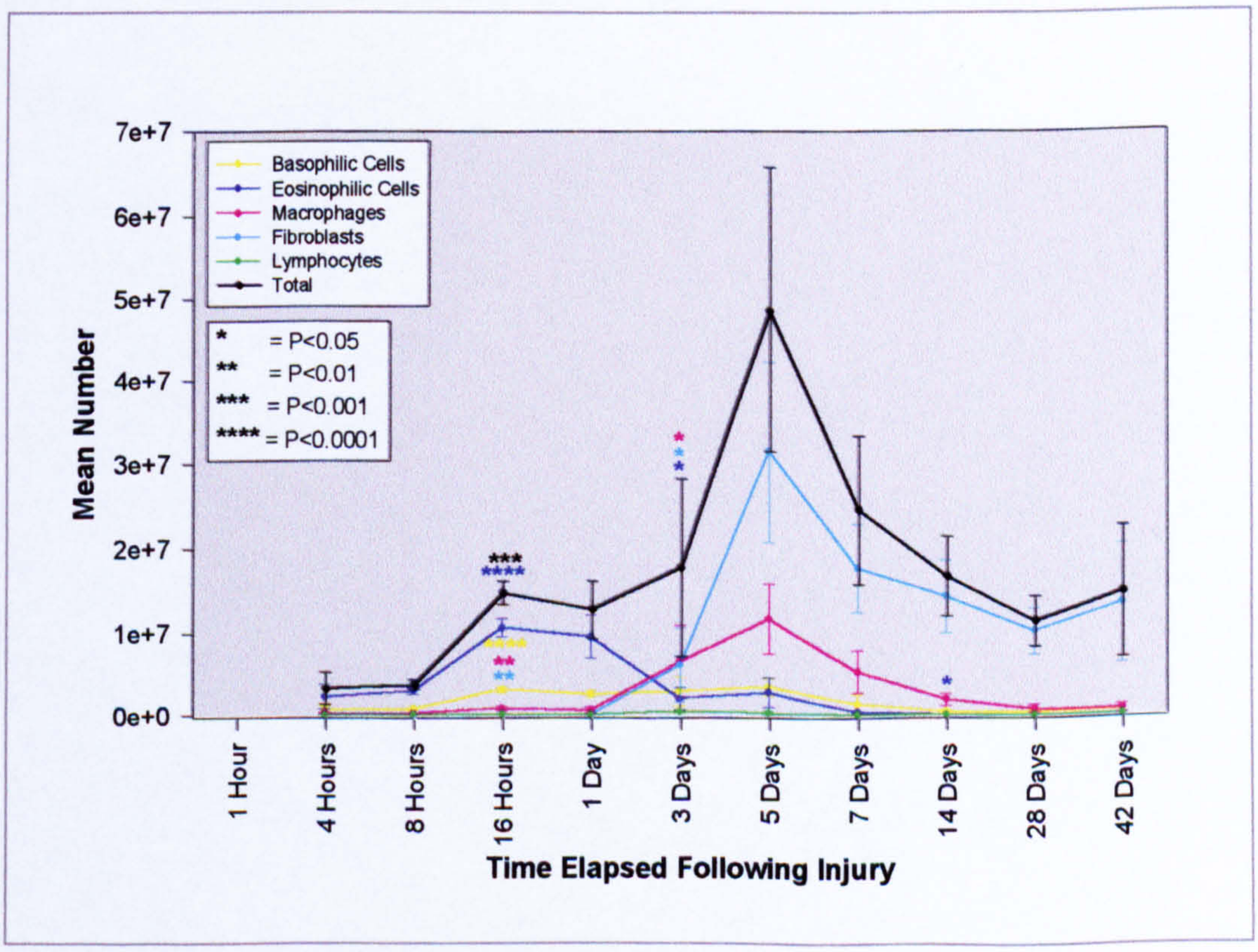
3.6.1.2.2 Post Surgical Adhesions (PSAs)

Total numbers of all cells analysed (total cells) located within PSAs through their formation and maturation demonstrated a similar bi-phasic pattern as seen with number density. There was an increase in mean number from 4 to 16 hours, with a significant increase ($P<0.001$) between 8 and 16 hours. Little change was seen from 16 hours to 3 days after which there was a rapid increase with mean numbers peaking at 5 days. From there on mean number of total cells decreased (Graph 3.19; Appendix III Tables 3.13 and 3.14).

Total numbers of basophilic cells in PSAs increased from 4 to 16 hours (Graph 3.19; Appendix III Tables 3.13 and 3.14). From 16 hours to 5 days mean number remained relatively constant with a decrease there after. Whereas eosinophilic cells demonstrated an increase from 4 to 16 hours, being significant ($P<0.0001$) from 8 to 16 hours. Little

change was seen from 16 hours to 1 day after which there was a decrease in mean numbers up until 28 days. This was significant ($P<0.05$) between 1 and 3 days and between 7 and 14 days. Mean numbers remained minimal from 28 to 42 days post injury (Graph 3.19; Appendix III Tables 3.13 and 3.14).

Macrophage numbers within PSAs remained unchanged from 4 hours to 1 day (Graph 3.19; Appendix III Tables 3.13 and 3.14). This was followed by a steady increase, significant ($P<0.05$) between 3 and 5 days, with mean numbers peaking at 5 days post injury and decreasing there after. Fibroblasts followed a similar pattern to macrophages. No significant change in mean number was seen within the first day however from 1 to 5 days there was a rapid increase, with a significant increase ($P<0.05$) between 3 and 5 days. After this 5 day peak levels decreased until the completion of the study (Graph 3.19; Appendix III Tables 3.13 and 3.14). Lymphocytes showed little variation throughout the course of this study, with a slight peak seen at 16 hours post injury (Graph 3.19; Appendix III Tables 3.13 and 3.14).



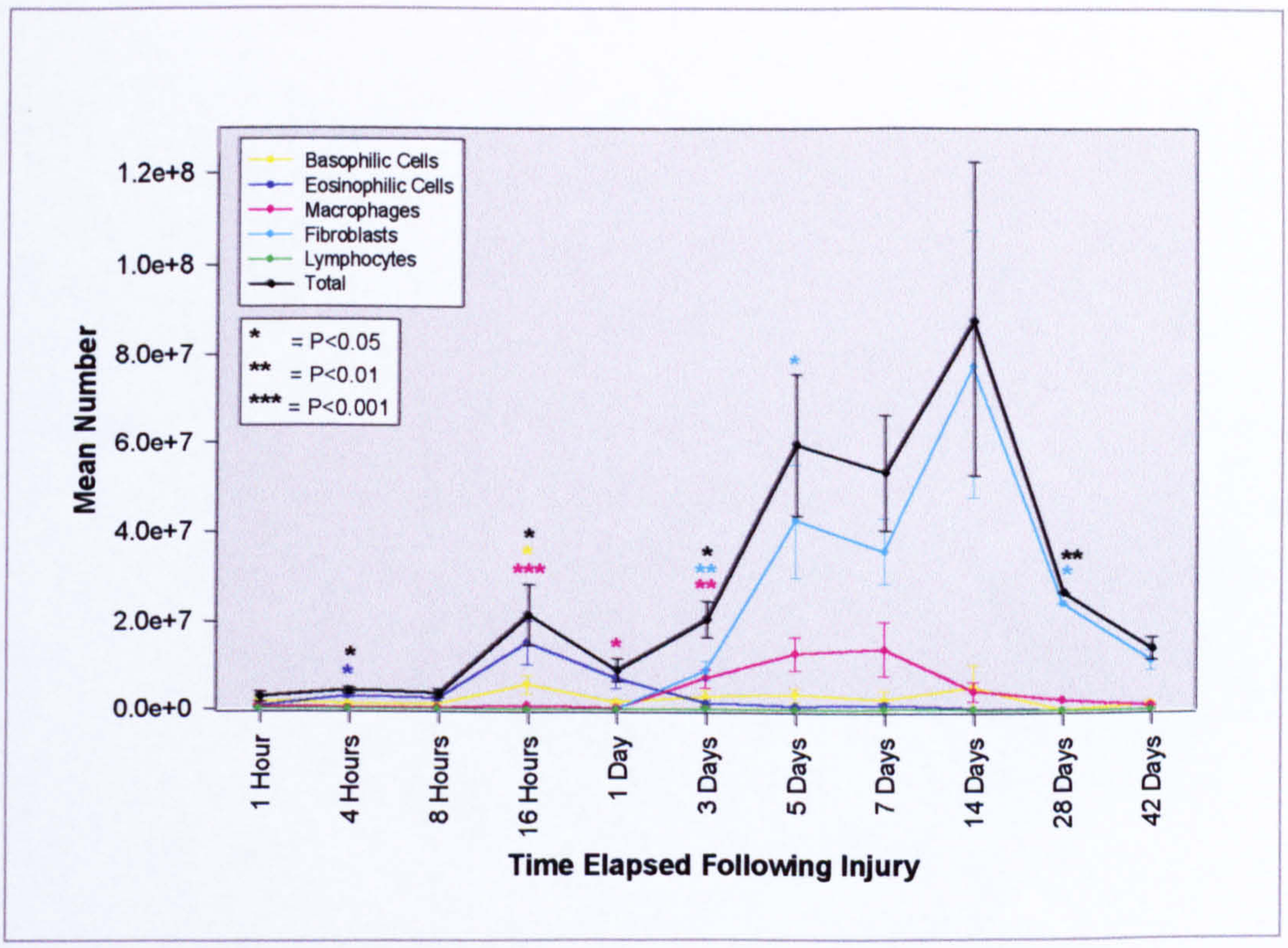
Graph 3.19: Mean cell numbers throughout PSA development together with SEMs and statistical differences.

3.6.1.2.3 Peritoneal Tissue Generation (PTG)

Total numbers of cells within PTG increased from 1 to 16 hours, with significant increases ($P<0.05$) from 1 to 4 and 8 to 16 hours post injury (Graph 3.20; Appendix III Tables 3.13 and 3.14). There was a decrease from 16 hours to 1 day although this was not statistically significant ($P>0.05$). From 1 to 14 days mean total numbers of cells increased, a significant increase ($P<0.05$) between 1 and 3 days. Following the peak at 14 days mean numbers rapidly decreased, with a significant decrease ($P<0.01$) between 14 and 28 days.

Numbers of basophilic cells increased from 1 hour to peak at 16 hours with a significant increase ($P<0.05$) seen between 8 and 16 hours. This was followed by comparatively constant number from 16 hours to 14 days, a decrease seen from there on (Graph 3.20; Appendix III Tables 3.13 and 3.14). Eosinophilic cells within PTG increased from 1 hour, a significant increase ($P<0.05$) between 1 and 4 hours, peaking at 16 hours post injury (Graph 3.20; Appendix III Tables 3.13 and 3.14). Mean numbers of eosinophilic cells then decreased to 3 days, with constant levels seen until the end of the study.

Macrophage numbers showed little variation for the first day (Graph 3.20; Appendix III Tables 3.13 and 3.14). However from 1 day there was a steady increase, significant between 1 and 3 days ($P<0.01$) with numbers peaking at 7 days post injury and decreasing there after. Numbers of fibroblasts demonstrated similar trends to macrophages. From 1 hour to 1 day there were no significant changes, however from 1 to 14 days mean numbers increased with significant increases seen between 1 and 3 ($P<0.01$) and 3 and 5 days ($P<0.05$). From 14 days onwards numbers of fibroblasts decreased, significantly ($P<0.05$) between 14 and 28 days (Graph 3.20; Appendix III Tables 3.13 and 3.14). Numbers of lymphocytes showed no significant trends throughout PTG formation and maturation (Graph 3.20; Appendix III Tables 3.13 and 3.14).



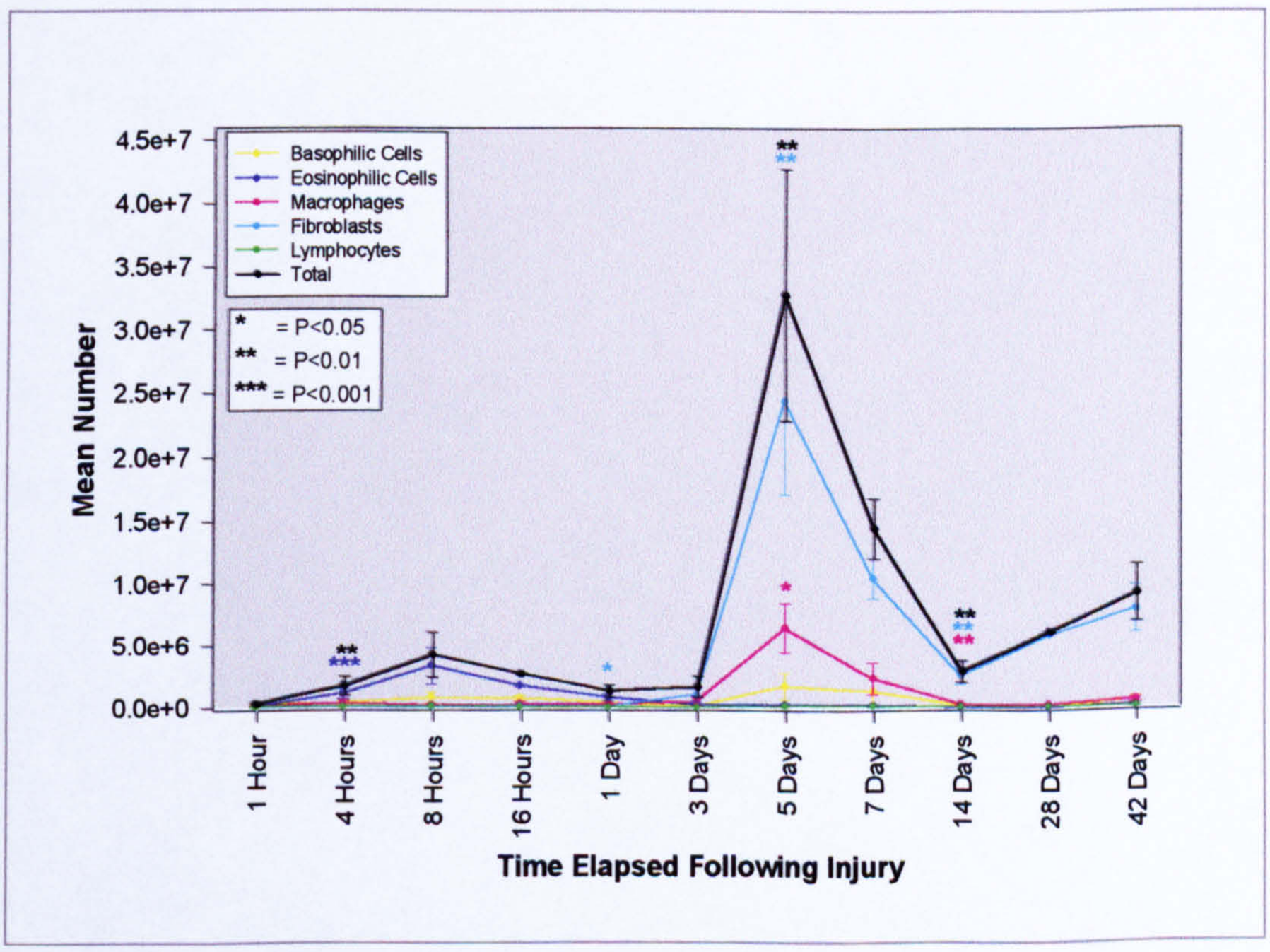
Graph 3.20: Mean cell numbers throughout PTG development together with SEMs and statistical differences.

3.6.1.2.4 Uterine Horn Tissue Generation (UTG)

Total numbers of cells analysed within UTG increased from 1 to 8 hours, with a significant increase ($P<0.01$) between 1 and 4 hours (Graph 3.21; Appendix III Tables 3.13 and 3.14). Little change was seen between 8 hours and 3 days however a significant increase ($P<0.01$) was seen between 3 and 5 days, with numbers peaking at this point. A rapid decrease in total number was demonstrated between 5 and 14 days, being significant ($P<0.01$) from 7 to 14 days. A trend to increase was subsequently seen from 14 days until the completion of this study.

Basophilic cells demonstrated an increase in number from 1 to 8 hours (Graph 3.21; Appendix III Tables 3.13 and 3.14). From 8 hours to 3 day little change was seen. However there was an increase from 3 to 5 days, levels remaining slightly elevated from 5 to 7 days and then decreasing. Numbers of eosinophilic cells increased from 1 to 8 hours, with a significant increase ($P<0.001$) between 1 and 4 hours. From 8 hours to 3 days mean numbers decreased with little variation from 3 days post injury onwards (Graph 3.21; Appendix III Tables 3.13 and 3.14).

Numbers of macrophages and fibroblasts within UTG demonstrated very similar trends. From 1 hour to 3 days there were no major changes in mean total number. Significant increases were seen in both macrophage ($P<0.05$) and fibroblasts ($P<0.01$) from 3 days to peak at 5 days post injury. Mean numbers then decreased up to 14 days, with significant decreases ($P<0.01$) between 7 and 14 days. Macrophages then remained relatively constant for the rest of the study. Fibroblasts, however, showed a trend for numbers to increase from 14 to 42 days (Graph 3.21; Appendix III Tables 3.13 and 3.14). Mean numbers of lymphocytes within UTG showed minimal levels through the course of this study, however slightly elevated numbers were seen from 5 to 7 days (Graph 3.21; Appendix III Tables 3.13 and 3.14).



Graph 3.21: Mean cell numbers throughout UTG development together with SEMs and statistical differences.

During the course of this study significantly greater numbers of cells were located in PTG compared to PSA at the following times: macrophages at 8 hours ($P<0.01$); total cells at 7 days ($P<0.05$); and fibroblasts and total cells at 14 days ($P<0.01$). Greater numbers of cells were found in PSA compared to UTG at: 16 hours for basophilic cells

(P<0.05), eosinophilic cells (P<0.01) and total cells (P<0.01); 1 day for basophilic cells (P<0.01), eosinophilic cells (P<0.01), lymphocytes (P<0.05) and total cells (P<0.01); and at 3 days (P<0.05) for basophilic cells, macrophages and total cells. Finally greater numbers of cells were located in PTG compared to UTG at: 1 hour for basophilic cells (P<0.05), eosinophilic cells (P<0.01), macrophages (P<0.01) and totals (P<0.001); 4 hours for basophilic cells (P<0.05), eosinophilic cells (P<0.01) and totals (P<0.05); 1 day (P<0.05) for basophilic, eosinophilic and total cells; 3, 7 and 14 days for macrophages (P<0.05), fibroblasts (P<0.05) and total cells (P<0.01); and 28 days for macrophages (P<0.05) (Appendix III Table 3.15).

The coefficient of error for number density counting was calculated to be 13%.

3.6.2 Discussion

Overall trends seen for cellular presence in developing PSAs and tissue generation were similar: From 1 hour to 1 day, inflammatory cells were the predominant cell types (eosinophilic cells, basophilic cells and macrophages respectively), however, from 3/5 days onwards tissue repair cells were the predominant cells present (fibroblasts and macrophages). Hence the formation of PSAs and tissue generation has two distinct cellular cycles: (i) inflammatory response - predominant up to 1 day post injury; and (ii) tissue repair response - predominant from 3 days onwards, with a rapid conversion between these two cellular processes.

The general trend in all tissue zones analysed for total cells present was an increase in mean number from 1 hour to 16 hours, with a peak at this point. This peak was due to a peak in eosinophilic and basophilic cells. This was followed by a decrease in mean number and subsequent increase to peak for a second time at 5 day post injury, due to a peak in fibroblast and macrophages. This in turn was followed by a decrease with mean numbers remaining consistent from 7 to 42 days. Hence during PSA and tissue generation development there are two distinct peaks in cellular presence: (i) 16 hours - a result of maximal presence of inflammatory cells (eosinophilic and basophilic cells); and

(ii) 5 days - due to maximal presence of tissue repair cells (fibroblasts and macrophages).

It has previously been established that both adhesion free healing and PSA formation have the same basic cellular profiles: haemostasis followed by the inflammatory response and finally tissue repair (refer to Chapter 1 of this thesis). The results of this study conform to this theory and lend support for the 1974 study by Milligan & Raftery. They determined that during the first day of PSA development, PMNLs were the predominant cells with additional macrophages and eosinophils also present. At 2 days PMNL predominance was shared with macrophages, however, eosinophils and lymphocytes were also detected. Between 2 and 3 days numbers of PMNLs decreased significantly with macrophages the most numerous cell with some eosinophils still present. From 4 to 7 days macrophages and eosinophils still remained although there were ever increasing numbers of fibroblasts. By 14 days there were relatively few cells present, with those consisting of elongated fibroblasts and occasional macrophages.

Differences seen with regard to PMNLs and eosinophil presence can largely be attributed to the animal species difference between this and Milligan & Raftery's study (1974). Their study utilised a rat model, which has easily defined and identifiable neutrophils and eosinophils. Whereas, as discussed in the Section 3.7 of this thesis, rabbit subsets of polymorphonucellular cells are less defined.

This study highlights that this transition between the inflammatory response and tissue repair with regard to cellular activity is very rapid and highly specific. Considering that the formation of PSAs is such a complex and multifactoral process such strict regulation is not surprising. For although PSA formation is sometimes regarded as "wound healing gone wrong", it is actually a well regulated, tightly controlled process.

Following the induction of injury, haemostasis will be initiated instantaneously to limit blood loss and tissue damage. The final outcome of this process is blood vessel contraction, the formation of a platelet plug and formation of a fibrin clot. Platelets entrapped within the platelet plug actively secrete chemical mediators which initiate the

inflammatory process, resulting in the escape of oedema fluid, including some plasma proteins and leukocyte migration.

Many different subsets of leukocytes are attracted to the chemotactic chemicals released at the injury site but their arrival is dependent upon their location within the body and their motility. Although leukocytes are primarily located within the circulatory system and within tissues, neutrophils (basophilic cells) have the advantages of being loosely adhered to vascular endothelium and are highly motile, with their migration accelerated by damage to blood vessels and inflammation. These are the first leukocytes present in any numbers at the site of injury as detailed in this and other studies. Eosinophils (eosinophilic cells) are the next cell type to arrive, with their predominance established very quickly (within 4 hours in this study). Both basophilic and eosinophilic cells possess similar characteristics, that is, they are capable of bacterial killing, phagocytosis and release of a whole host of cytokines and growth factors, eosinophils are also thought to possess anti-histamine properties which have been hypothesised as trying to limit the formation of PSAs (Milligan & Raftery, 1974) and be important in the organisation of wound healing.

Following this period of intense inflammatory mediation many cytokines and growth factors are synthesised and released, which act as further attractants to cells. Mononuclear leukocytes are present both in blood as monocytes or in tissues as macrophages. The blood monocyte is well established as sluggishly motile hence its arrival at the site of injury is after basophilic and eosinophilic cells. Tissue macrophages actively kill bacteria and are highly phagocytotic - removing tissue debris and necrotic tissue from the injury site in order for proper wound healing to follow. Macrophages also release a wide variety of cytokines and growth factors, acting as potent stimulators enhancing trauma cell proliferation and angiogenesis.

The final cell which is essential for wound healing and PSA formation is the fibroblast. Once present at the injury site, fibroblasts proliferate and synthesise collagen forming a protective layer over injured tissues and establishing fibrous PSAs. They release a variety

of chemical mediators and play a pivotal role in angiogenesis. By 5 to 7 days following injury, numbers of fibroblasts declined to a constant level, due to fibroblasts becoming trapped in the collagen matrix.

Lymphocyte presence within PSAs or tissue generation throughout their development showed little change. Hence no case in this study developed any significant level of infection which would have required an immune reaction mounted against it. It is universally accepted that infection is a potent stimulator of PSAs however this did not play a factor in the formation of the PSAs within this study.

Although both PSA and peritoneal and uterine horn tissue generation followed the same overall pattern with regard to cell predominances throughout their respective development, significant differences ($P<0.05$) were seen at several sample time points. At both 5 and 28 days there were greater number densities of fibroblasts and total cells within PSAs compared to PTG. This is thought to be a result of greater attraction of the fibroblasts into PSAs at 5 days, with their presence sustained for a longer length of time (demonstrated at 28 days). This could be due to fibroblast evacuation being more difficult within PSA tissue compared to PTG, or their greater numbers increasing evacuation time.

No significant differences ($P>0.05$) were seen between PSA and UTG number densities. However, between PTG and UTG tissues there were differences ($P<0.05$) at 1, 4 and 16 hours and 28 days. It is most likely that greater number densities were seen within UTG compared to PTG due to greater vascularity of the uterine horn compared to the peritoneal wall, allowing quicker and increased access to damaged uterine horn tissue compared to damaged peritoneum tissue.

Number totals within PSAs demonstrated peaks at 16 hours, a result of a peak in number densities of inflammatory cells and increase in PSA volume and a peak at 5 days, resulting from a peak in number density of tissue repair cells and PSA volume. The peak in inflammatory cells corresponds to lay down of fibrin, that is, PSA modelling and the

peak in tissue repair cells to maximum lay down of collagen, that is, PSA remodelling.

Peritoneal and uterine horn tissue generation demonstrated peaks in total numbers of cells at 16 and 8 hours respectively. These were the primary result of the peaks in number density of inflammatory cells, with little to no influence from tissue generation volume. The second peak in number totals seen at 14 days for PTG was due to peak in PTG volume rather than an increase in number densities whereas the peak at 5 days for UTG was due to peaks in UTG volume and number densities. Thus the peak in inflammatory cells within tissue generation had no noticeable effect on the volume of fibrinous tissue generated, which is in contrast to the situation seen within PSAs. The peak in tissue repair cells demonstrated an effect of increasing tissue generated although this was a lesser extent in PTG compared to either PSA or UTG. Differences seen between PSA and tissue generation with regard to the response to inflammatory cell presence could be due to: (i) differences in number densities and totals within PSA/PTG/UTG; and/or (ii) PSA inflammatory cells being more "biochemically" active.

Conclusions

i. PSA, PTG and UTG demonstrated similar trends with regard to predominant cell types throughout their development: inflammatory cells (eosinophilic cells, basophilic cells and macrophages) predominant from 1 hour to 1 days, with tissue repair cells (fibroblasts and macrophages) predominant from 3 days onwards.
ii. Peak in inflammatory cells were seen at 16 hours post injury, with the tissue repair cell peak at 5 days.
iii. For PSAs, the inflammatory peak resulted in PSA modelling, however for PTG and UTG the inflammatory peak demonstrated no physiological effect.
iv. The peak in tissue repair cells resulted in an increased volume of PSA and tissue generation, although this effect was reduced within peritoneal tissue.

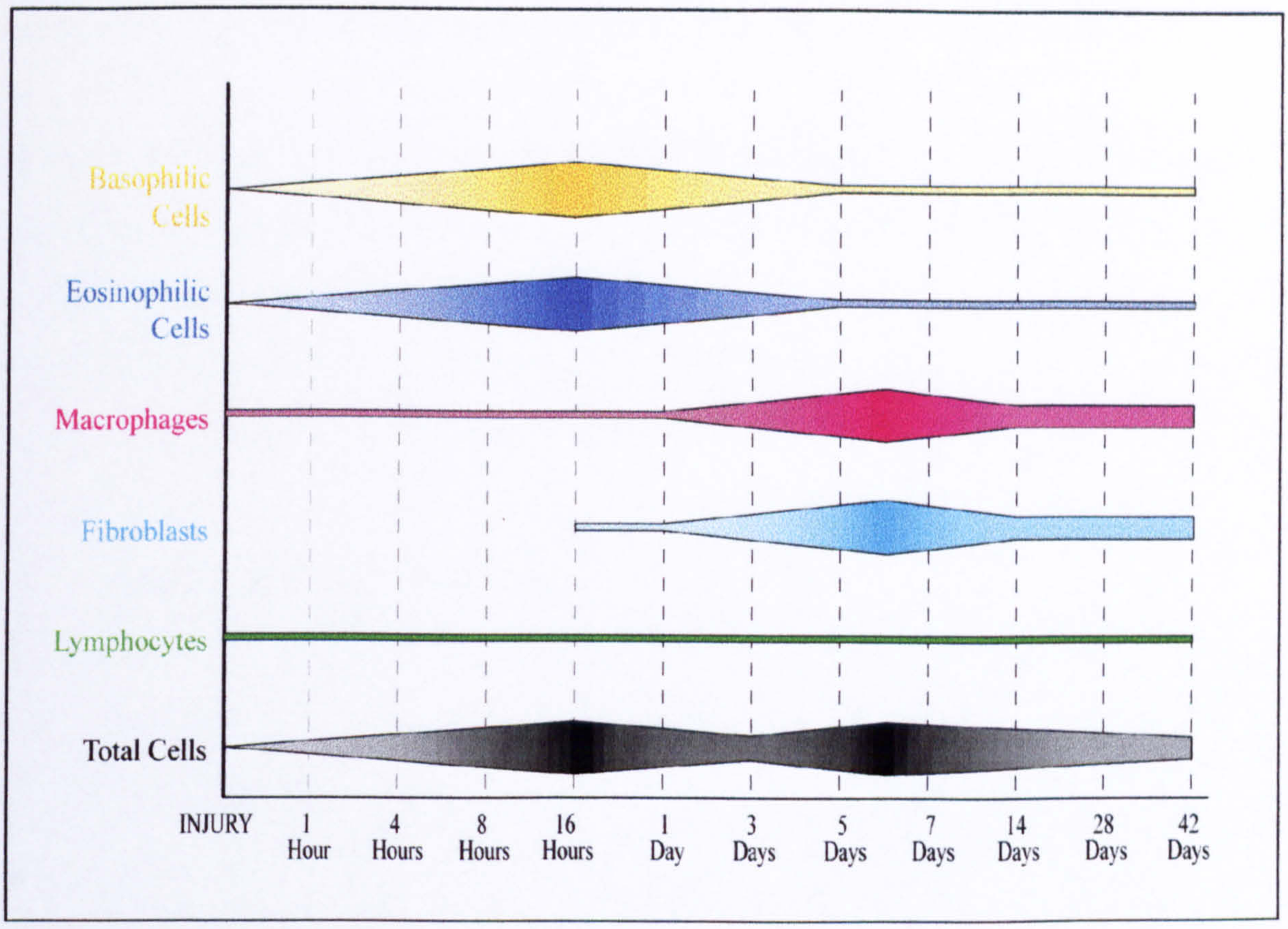


Figure 3.14: Summary of cellular presence within tissue generated.

3.7 Biochemical Analyses

Formation of PSAs is a complex, dynamic process, involving cellular and biochemical systems. Although this activity occurs locally the possibility of systemic involvement has as yet remained uninvestigated. The aim of these experiments was to study any systemic changes in blood cell numbers and tumour necrosis factor alpha (TNF-α) and transforming growth factor beta (TGF-β) (in both plasma and peritoneal fluid).

Blood samples were taken at three points throughout the experimental procedure:

i. blood sample 1: taken pre-operative, that is, following anaesthesia but before any surgical procedure. This sample acts as both an intra-case control and overall control for our animals in this study.
ii. blood sample 2: taken immediately following initial laparotomy. This sample aims to identify any change in response to opening the peritoneal cavity.
iii. blood sample 3: taken after the second (termination) laparotomy, with the aim of

identifying systemic changes occurring with wound healing and/or PSA development.

Peritoneal fluid samples were taken at two different points throughout the procedure:

i. peritoneal fluid sample 1: taken immediately following the initial laparotomy to act as a (laparotomy) control sample.
ii. peritoneal fluid sample 2: taken following the second (termination) laparotomy to identify any changes associated with wound healing and/or PSA development.

3.7.1 Full and Differential Blood Counts

All mammals have similar blood cell types: red blood cells (RBCs), platelets, and white blood cells (WBCs), with the latter further differentiated into neutrophils, eosinophils, basophils, monocytes and lymphocytes (refer to Appendix I Table 1.2 for cell functions). Morphological differences exist between human and several mammalian species, with the main difference between rabbit (Appendix III Table 3.16) and human blood cells is that rabbit neutrophils contain granules which stain pink, that is eosinophilic granules, which are absent in human neutrophils. This difference has lead to confusion with cell identification of rabbit blood cells over the years. For the purpose of this study, granular cells which contained eosinophil granules were termed eosinophilic cells and those with only basophil granules termed basophilic cells. In addition monocytes and lymphocytes were counted.

Most routine human full blood counts are performed using automated technology due to the time consuming nature of manual counts and possible inaccuracies. For this study both automated and manual counts were carried out due to the automated analyser used being set up for human samples, hence will fail to identify differences between rabbit and human blood cells, producing incorrect differential counts. Results reported in this thesis for RBCs, platelets and total WBCs are taken from the auto-analyser with WBC differential counts results from manual counting methods.

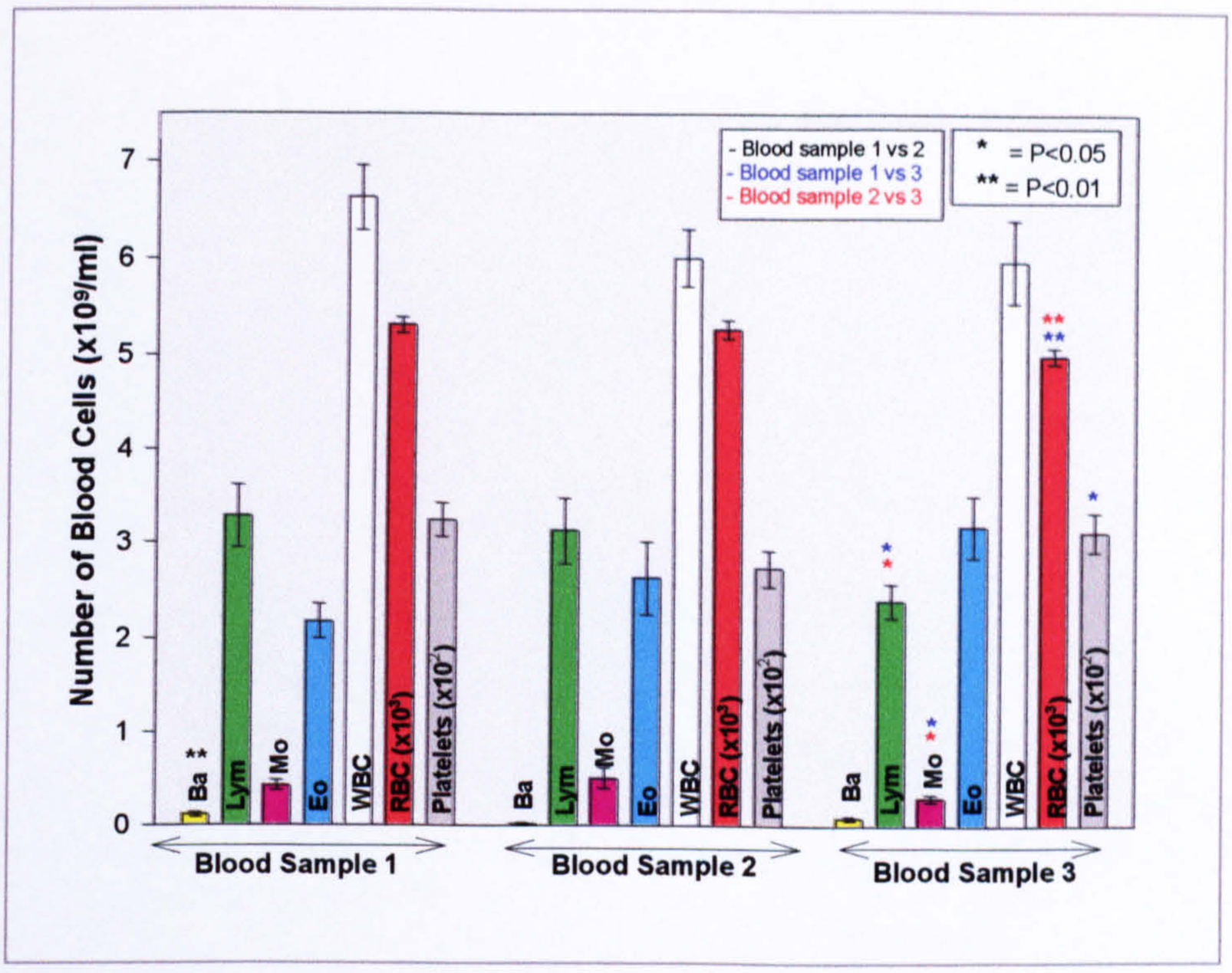
3.7.1.1 Results

Analysis of blood sample 1 (pre-operative) showed mean number of RBCs to be 5.31 $x10^{12}$/l, platelets 324 $x10^{9}$/l, and WBCs 6.62 $x10^{9}$/l. Differential counts showed 0.11 $x10^{9}$/l basophilic cells, 3.28 $x10^{9}$/l lymphocytes, 0.42 $x10^{9}$/l monocytes and 2.18 $x10^{9}$/l eosinophilic cells (Graph 3.22; Appendix III Table 3.17).

Mean numbers of cells at blood sample 2 (post-laparotomy) were 5.27 $x10^{12}$/l RBCs, 273 $x10^{9}$/l platelets, 6.00 $x10^{9}$/l WBCs, with 0.02 $x10^{9}$/l basophilic cells, 3.13 $x10^{9}$/l lymphocytes, 0.51 $x10^{9}$/l monocytes and 2.63 $x10^{9}$/l eosinophilic cells (Graph 3.22; Appendix III Table 3.17).

Overall mean numbers of cells at blood sample 3 (pre-termination) was 4.99 $x10^{12}$/l RBCs, 311 $x10^{9}$/l platelets, 5.96 $x10^{9}$/l WBCs, with 0.08 $x10^{9}$/l basophilic cells, 2.39 $x10^{9}$/l lymphocytes, 0.30 $x10^{9}$/l monocytes and 3.19 $x10^{9}$/l eosinophilic cells (Graph 3.22; Appendix III Table 3.17).

Statistical analysis of mean numbers for each cell type between blood samples 1 and 2 showed a significant difference in basophilic numbers only (P=0.0083). Analysis between blood samples 1 and 3 demonstrated significant differences for lymphocytes (P=0.0164), monocytes (P=0.0159), RBCs (P=0.0064) and platelets (P=0.0137). Whereas analysis of blood samples 2 and 3 showed significant differences for lymphocytes (P=0.0415), macrophages (0.0292) and RBCs (P=0.0051) (Graph 3.22; Appendix III Table 3.18).

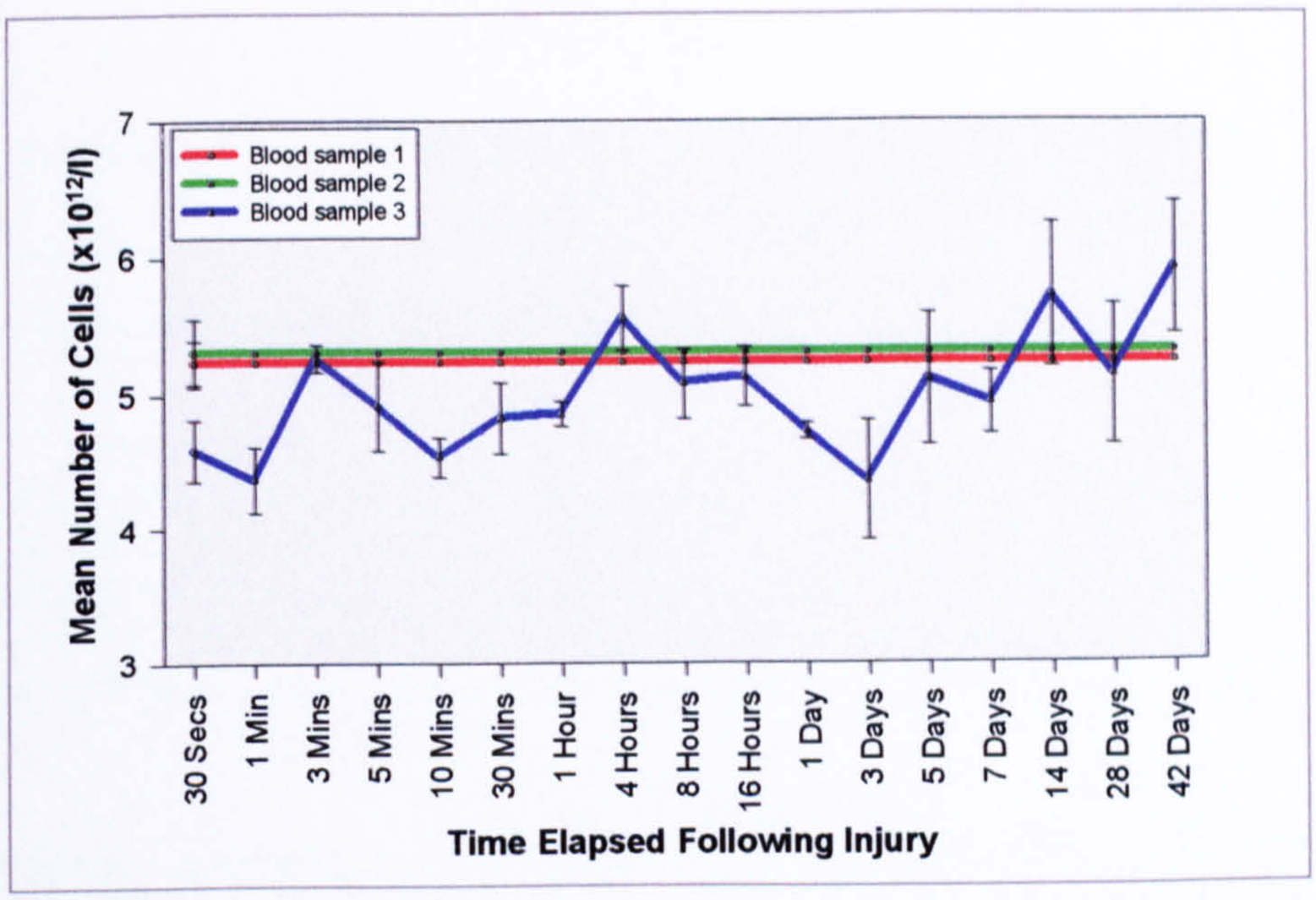


Graph 3.22: Mean numbers of blood cells for all blood samples SEMs and statistical differences.

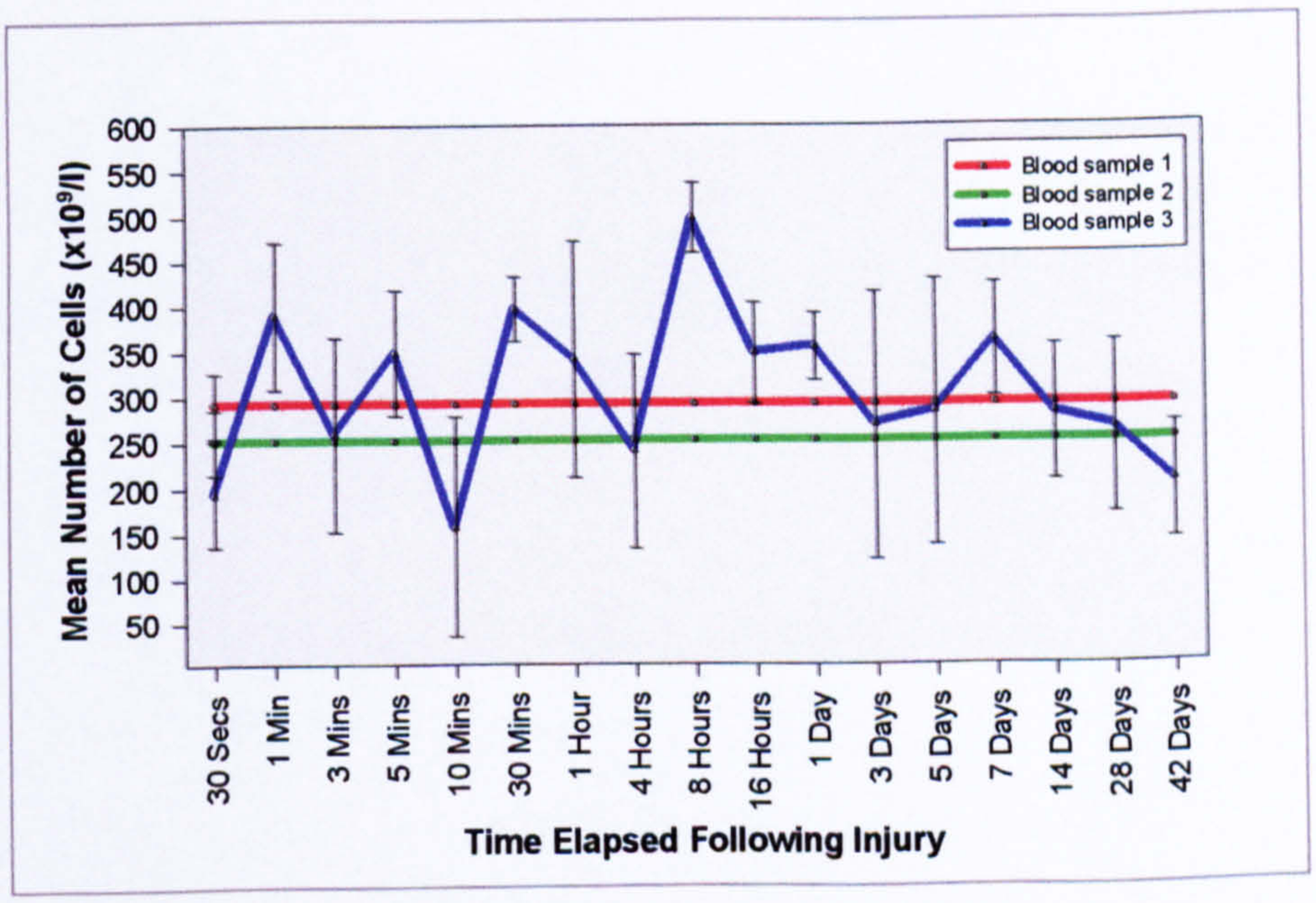
Note:

Ba = basophilic cells; Lym = lymphocytes;
Eo = eosinophilic cells; Mo = monocytes.

Blood samples 1 and 2 were control samples, however sample 3 was an experimental sample taken at time points throughout wound healing or PSA development (Appendix III Table 3.19). Mean numbers of both RBCs and platelets for blood sample 3 at each time point showed very little variation compared to control samples (blood sample 1 and 2) (Graph 3.23 and 3.24; Appendix III Table 3.19). However WBCs demonstrated differences in blood sample 3 compared to control samples (Graph 3.25; Appendix III Table 3.19). From 30 seconds to 1 hour mean numbers showed a trend to be lower in blood sample 3, with a rapid increase from 1 hour to a peak at 8 hours. Following this, mean numbers gradually decreased to control levels at 3 days post injury and WBC numbers remained consistent until the end of the study.



Graph 3.23: Mean RBC numbers for all blood samples together with SEMs.



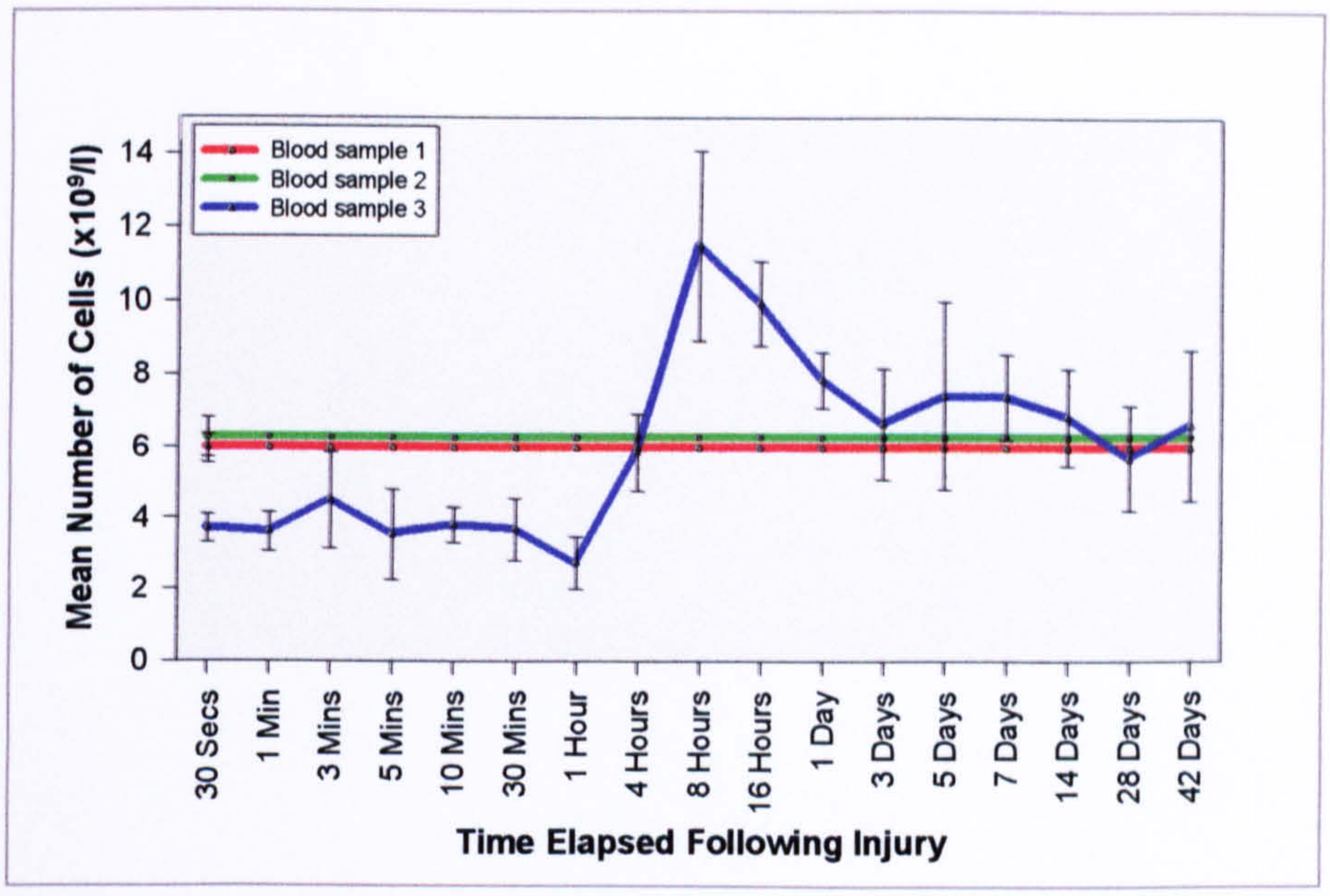
Graph 3.24: Mean numbers of platelets for all blood samples together with SEMs.

Basophilic cells demonstrated a trend to remain consistently low up to 3 days post injury, with levels similar to blood sample 2. After 3 days mean numbers increased and remained elevated between 5 and 14 days. Following this time point there was a trend for mean numbers to decrease (Graph 3.26; Appendix III Table 3.19).

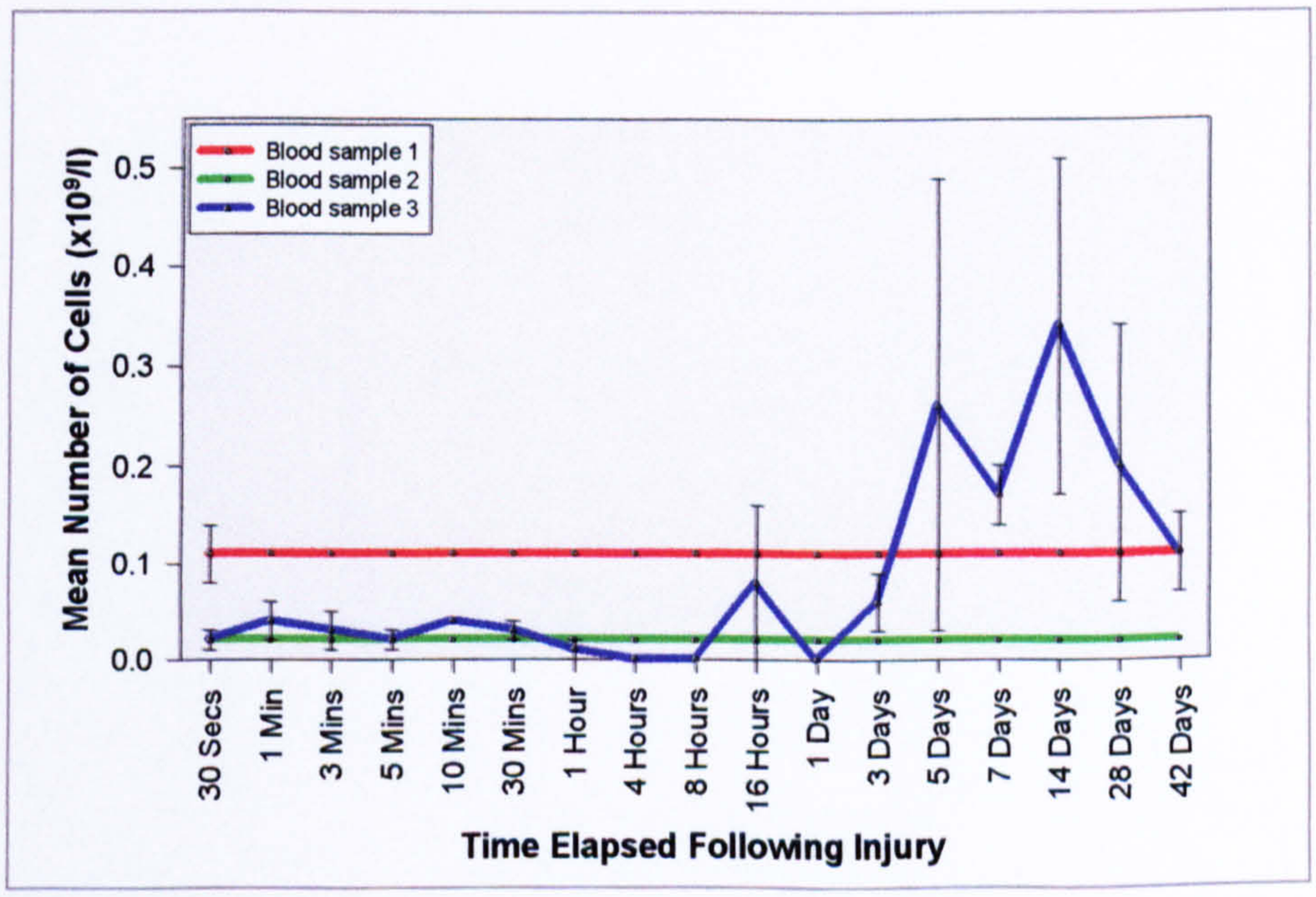
Mean numbers of lymphocytes showed a trend to decrease up to 30 minutes post injury with subsequent increases up to 3 days (Graph 3.27; Appendix III Table 3.19). From thereon mean numbers decreased to settle out at control levels from 5 days onwards.

Mean numbers of monocytes for blood sample 3 demonstrated consistently reduced levels compared to controls up to 3 days following injury, after which numbers increased to control numbers (Graph 3.28; Appendix III Table 3.19).

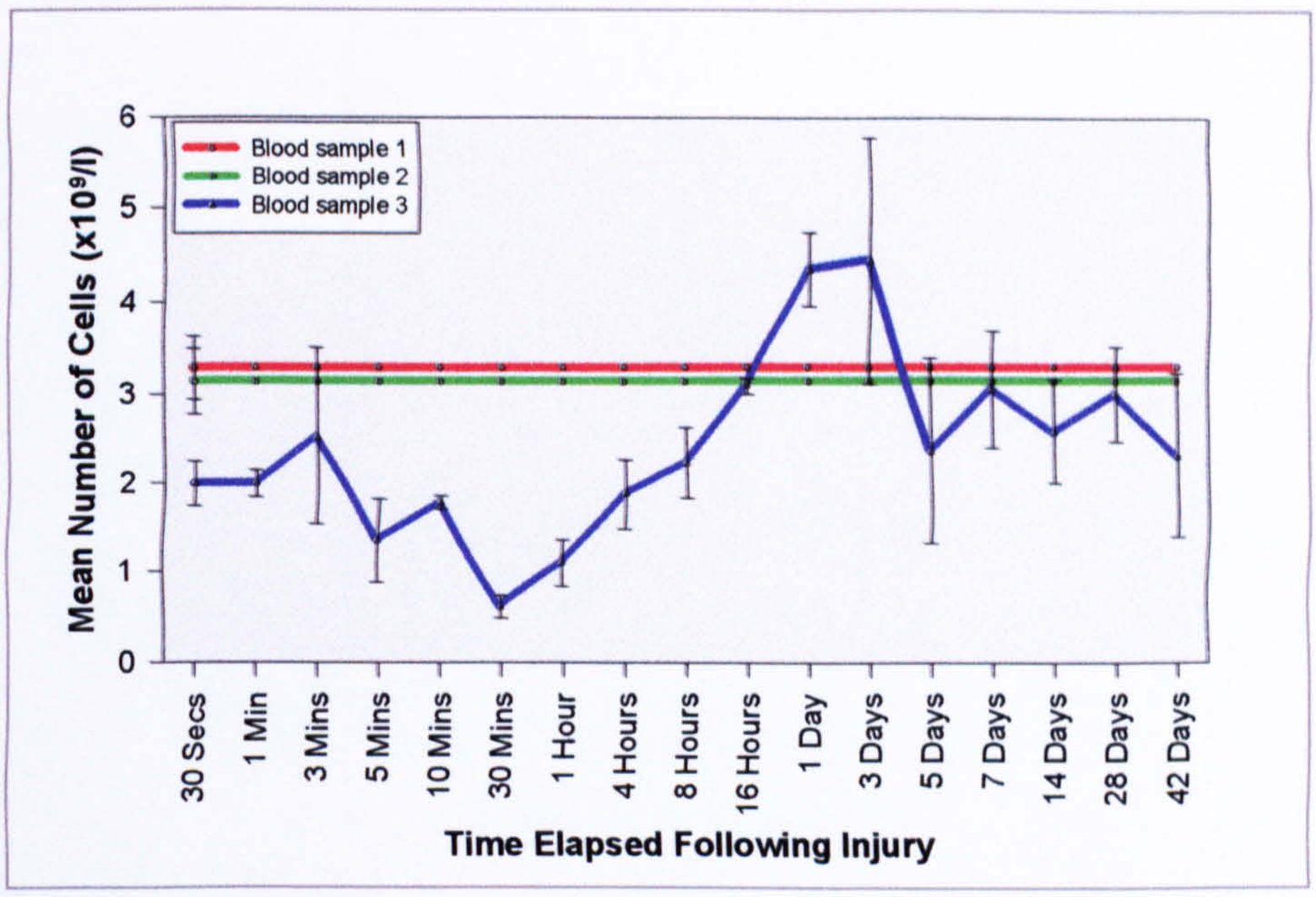
Eosinophilic cells demonstrated mean numbers similar to controls from 30 seconds to 1 hour (Graph 3.29; Appendix III Table 3.19). However, following this there was a rapid increase to a transient peak at 8 hours with numbers decreasing rapidly thereafter to 3 days post injury. From 3 to 42 days levels remained akin to controls.



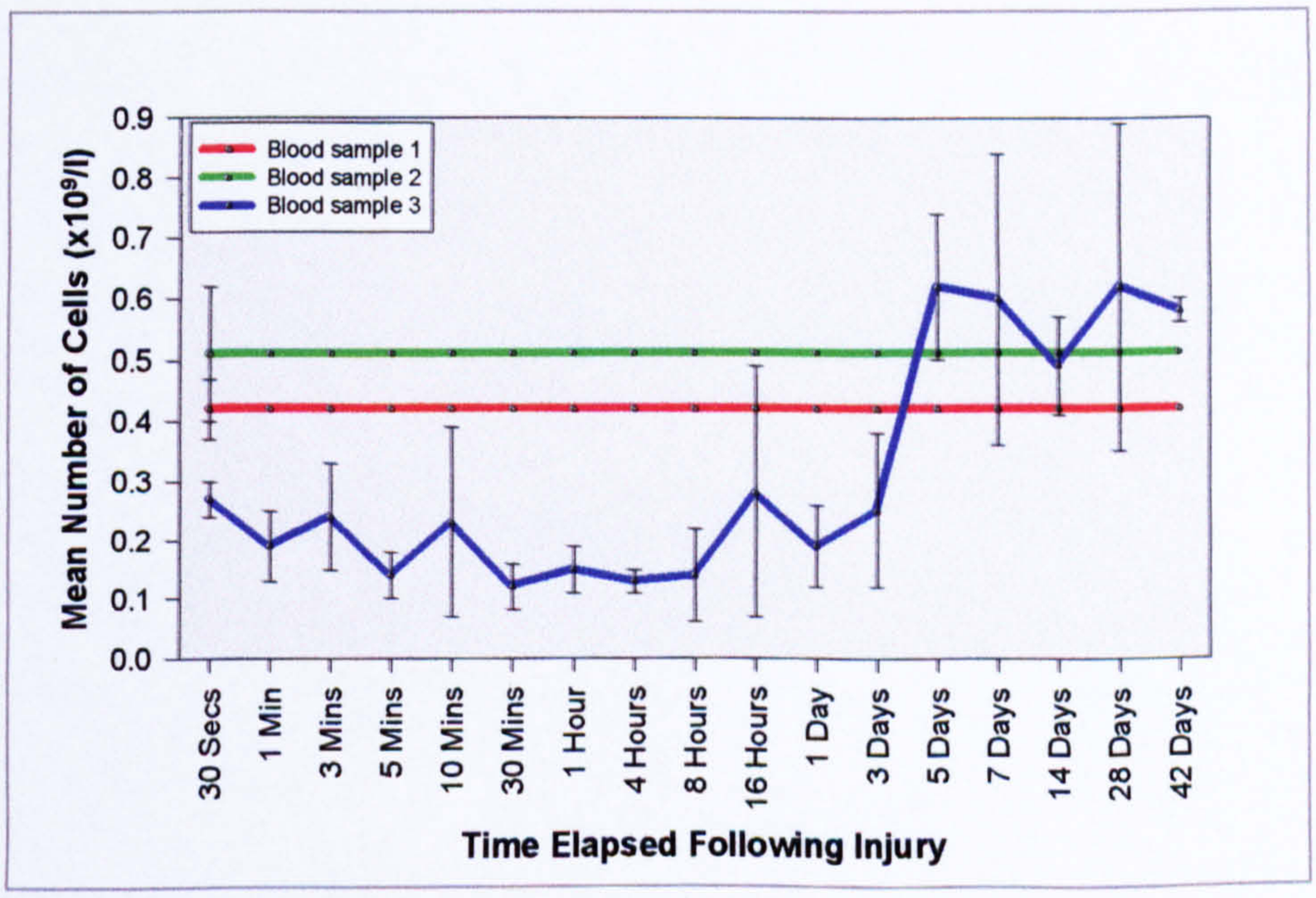
Graph 3.25: Mean numbers of WBCs for all blood samples together with SEMs.



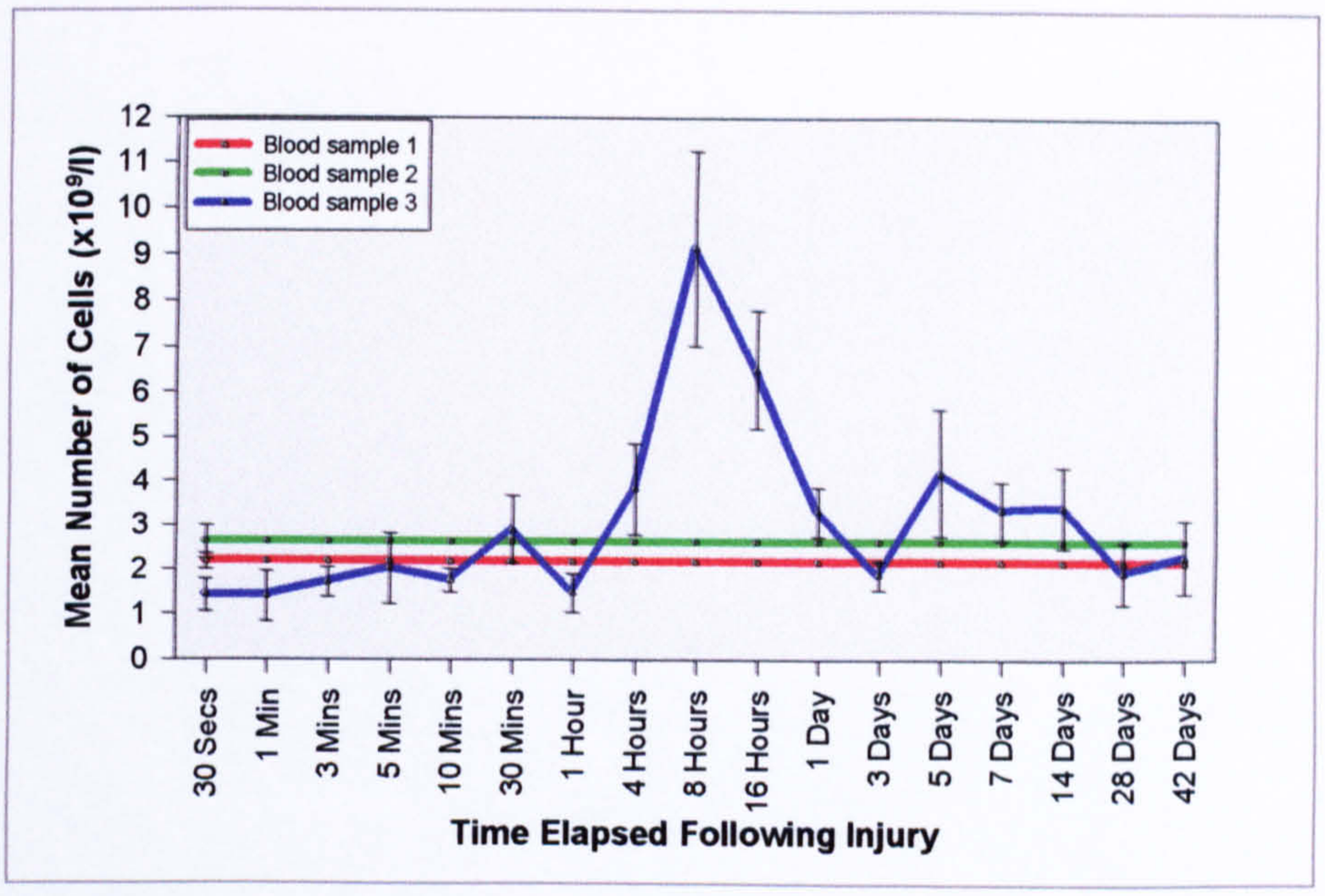
Graph 3.26: Mean numbers of basophilic cells for all blood samples together with SEMs.



Graph 3.27: Mean numbers of lymphocytes for all blood samples together with SEMs.



Graph 3.28: Mean numbers of monocytes for all blood samples together with SEMs.



Graph 3.29: Mean numbers of eosinophilic cells for all blood samples together with SEMs.

3.7.1.2 Discussion

Rabbits are frequently utilised in haematological studies due to their inappreciable fluctuations and ease of bleeding. However previously published data by various authors demonstrate marked differences which can be attributed not only to technical skill differences but also the unreliability of these animals with regard to blood factors (Schermer, 1967). Venesection also exerts considerable effects on blood cell profiles, especially when volumes of 10 to 20ml are removed, which are shown on Figure 3.15. During this study a 5ml blood sample was taken pre-operatively, 5ml taken post-laparotomy, with a final 5ml sample taken prior to termination. For the post-laparotomy (sample 2) and pre-termination (sample 3) samples effects of venesection need to be taken into consideration.

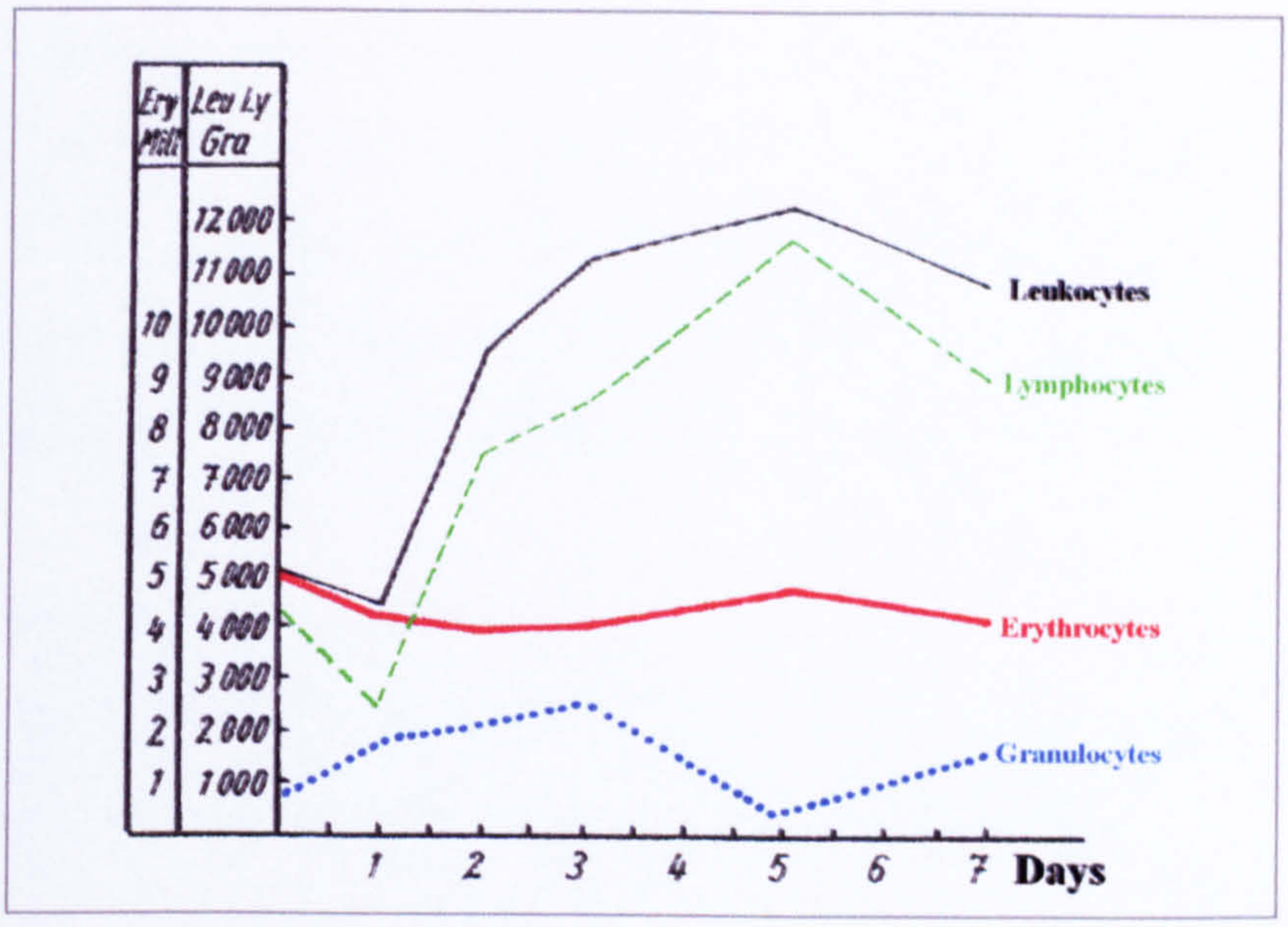


Figure 3.15: Rabbits response to venesection (20ml)
(Taken from Schermer, 1967).

Although several studies have investigated local cellular changes during adhesion-free healing and PSA development there is no published information on systemic cellular changes. Since marked local cellular and biochemical activity occurs throughout PSA formation it is feasible that cellular fluctuations are also demonstrated systemically.

Since the invasive technique of laparotomy was utilised in the induction of PSAs, any potential cellular changes could be attributed to laparotomy rather than PSA development. To identify such changes and eliminate them from PSA development, a blood sample was taken following the initial laparotomy in addition to a pre-operative control sample and experimental, PSA development, sample. Statistical analysis demonstrated only a significant difference between the pre-operative control samples and post-laparotomy sample for basophilic cells. Since very few basophilic cells were present this difference is most probably due to biological variation rather than a physiological response. Hence in this study there was little systemic cellular response to laparotomy. Further support for this comes from statistical analysis of control samples (pre-operative and post-laparotomy) with experimental samples, with significant differences ($P<0.05$) seen with the same cell types (Appendix III Table 3.17).

Total mean numbers of RBCs, platelets and WBCs for all blood samples taken fall within

normal ranges (refer to Appendix III Table 3.16). Since controversy exists with regard to rabbit WBC differentiation and identification it is only valid to compare lymphocyte and monocyte numbers, with results obtained in this study again falling with normal values. Overall it can be concluded that animals in this study provide a fair representation of normal haematology with regard to full and differential blood counts.

Due to the limited number of cases (generally 4) it was not valid to perform statistical analyses between experimental blood sample 3 and control samples at time points following injury. However identification of trends could be carried out.

There was little variation from control for both mean numbers of RBCs and platelets within experimental blood sample 3. Since RBCs are not involved in wound healing and platelets only involved in the formation of the platelet plug during haemostasis then their similarity to control blood counts was as expected.

However predominant trends were present for both total and differential counts of WBCs throughout PSA development. Total mean numbers of WBCs showed a trend to be lower than normal during the first hour following injury, with a rapid increase in numbers thereafter. By 3 days systemic levels of WBCs could be seen to be similar to controls, with this pattern continued until the end of the study. The pattern seen mimics that seen with the effects of venesection, hence it is probable that these changes are due to blood collection rather than wound healing/PSA development.

Mean numbers of basophilic cells demonstrated little variation during the first 3 days following injury, after which levels became elevated above normal values until 28 to 42 days. Although the pattern up to 5 to 7 days appears to correspond to that seen with venesection, elevated levels seen after these time points appears to be due to other factors. Basophilic cells are primarily involved in the inflammatory stage of wound healing therefore increased systemic levels at the latter end and completion of inflammation could be due to: (i) extended inflammatory period in this experimental model; (ii) basophilic cells involved in subsequent processes in this experimental model;

or (iii) over compensation in response to injury.

Systemic numbers of lymphocytes demonstrated a trend to be lower than controls from 30 seconds to 8 hours following injury. This depletion in systemic numbers was corrected by 16 hours, with slight peak in numbers at 1 and 3 days and levels normal thereafter. Although the time scale varies slightly from that seen with venesection a similar pattern is observed and venesection is probably responsible for changes seen.

Monocytes, highly phagocytic cells, also demonstrated consistently reduced levels from 30 seconds until 3 days following injury, indicating monocytes leaving circulation and entering injured tissues during the process of inflammation. It is not until inflammation is near completion that systemic mean numbers of monocytes return to control levels, highlighting their importance in wound healing. The effect of venesection was a mild increase in these cells after 1 day (Schermer, 1967) and hence does not appear to play an important role in the monocytic pattern seen during this study.

Eosinophilic cells demonstrated control values throughout the whole of this study, except for the large transient peak at 8 hours post injury. Eosinophils have been implemented in PSA development (Milligan & Raftery, 1974), with their predominance in tissues seen at 1 and 3 days. Thus there appears to be a recruitment of eosinophilic cells into circulation prior to maximum local presence. As with monocytes, venesection had the effect of causing a mild increase in eosinophilic cells after 1 day (Schermer, 1967) and again appears unimportant in this cell type.

Conclusions

i. In this study there was little to no systemic cellular response to laparotomy.
ii. Animals in this study provide a fair normal haematological representation with regard to full and differential blood counts.
iii. There was little variation from controls for both mean numbers of RBCs and platelets for experimental samples taken throughout PSA development.
iv. Predominant trends were present for both total and differential counts of WBCs

throughout PSA development, with some attributed to venesection and others PSA dependent. PSA associated changes were:

- elevated basophilic cell numbers at 14 and 28 days
- reduced numbers of monocytes from 30 seconds to 3 days
- transient peak in eosinophilic cell numbers at 8 hours.

v. Systemic cellular changes seen during this study should be interpreted cautiously due to the small numbers of samples at each time point, effects of venesection and the hypersensitivity and large variability of rabbit cell profiles.

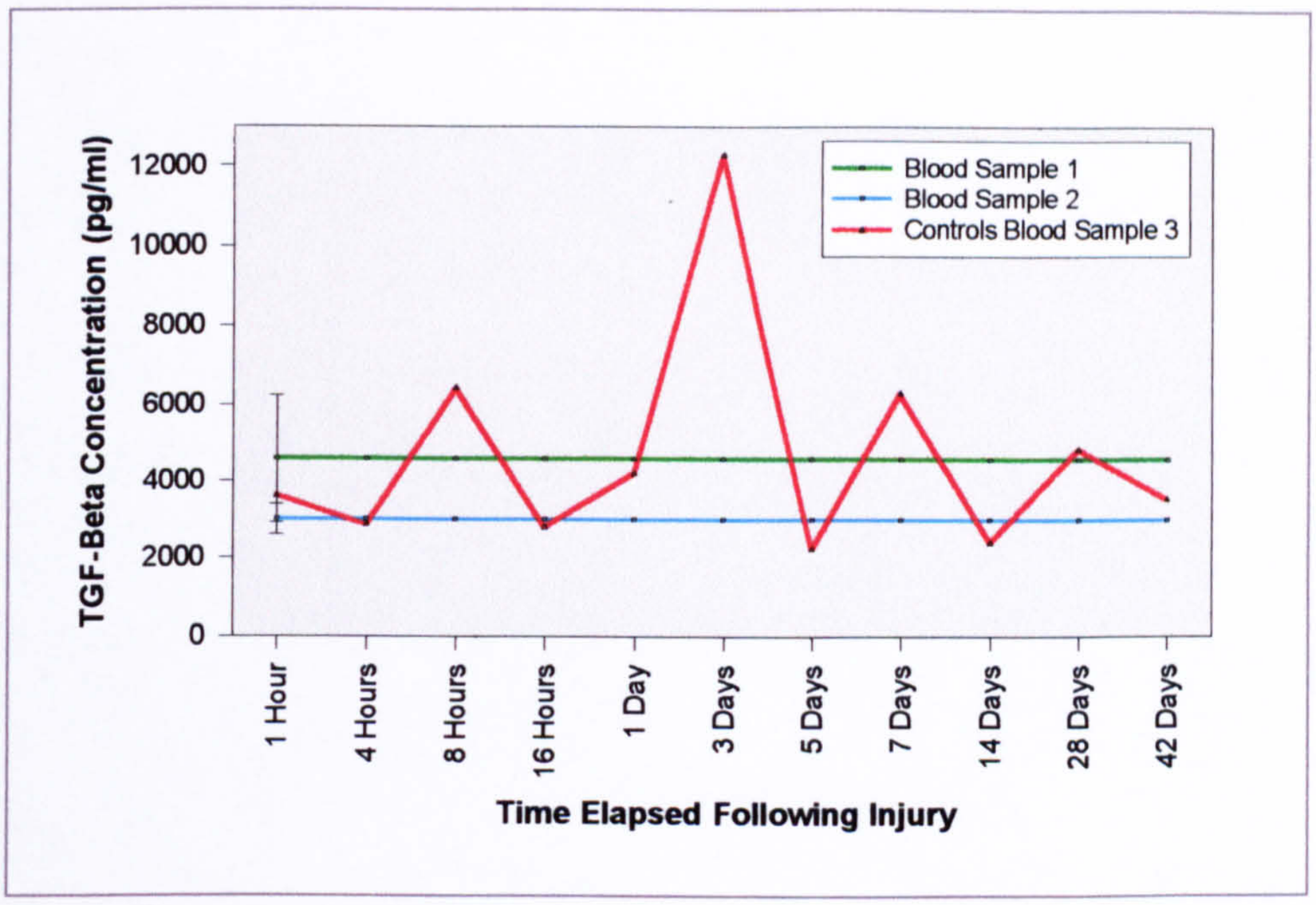
vi. PSA development demonstrated systemic changes with regard to WBCs, basophilic cells, monocytes and eosinophilic cells, primarily linked to the inflammatory stage of wound healing.

3.7.2 TGF-β Concentrations

The ELISA kits employed for detection of TNF-α (Genzyme) and TGF-β (Genzyme and R&D Systems) in plasma and peritoneal samples collected throughout this study were not specific for the detection of rabbit cytokines (refer to Chapter 2). After extensive experiments to determine accurate and reproducible detection of rabbit cytokines, only the R&D Systems TGF-β ELISA kit was deemed to be acceptable. Due to time constraints only one plasma from time points 1 hour to 42 days and only one undiluted peritoneal fluid sample from 5 to 28 days were assayed, together with controls.

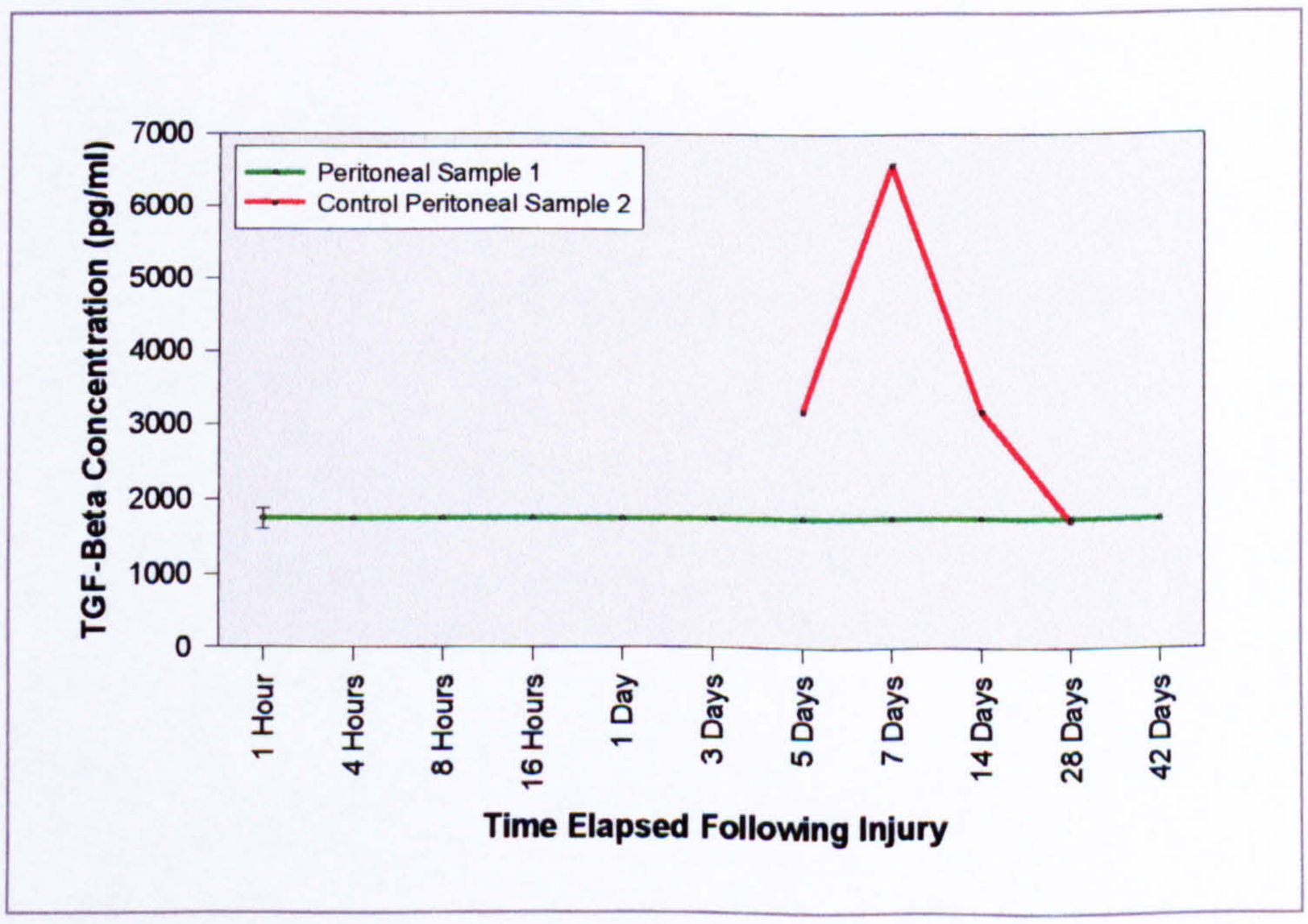
3.7.2.1 Results

Similar TGF-β concentrations were demonstrated in pre-operative control sample 1 and post-laparotomy sample 2, with 36% and 13% coefficient of variance within these samples respectively. With regard to experimental sample 3 fluctuations in TGF-β concentrations were seen throughout PSA development however these were all within control concentration except for a large peak at 3 days post injury (Graph 3.30; Appendix III Table 3.20)



Graph 3.30: TGF-β concentrations in blood samples.

TGF-β was detected in both post-laparotomy control peritoneal sample 1 and experimental peritoneal sample 2 (Graph 3.31; Appendix III Table 3.20). Control samples demonstrated small variability with 8% coefficient of variability whereas TGF-β concentrations in experimental samples showed a trend to peak at 7 days and subsequently decrease to control concentrations by 28 days post injury.



Graph 3.31: TGF-β concentration in peritoneal fluid samples.

3.7.2.2 Discussion

Due to the very limited number of samples assayed definitive conclusions can not be made. However it is possible to propose emerging trends.

All plasma and peritoneal fluid samples assayed demonstrated TGF-β present. Mean normal resting TGF-β concentrations in human serum and platelet poor plasma (EDTA) activated and assayed by R&D Systems were found to be 48.6ng/ml and 2.26ng/ml respectively. Direct comparisons between human and rabbit resting levels of TGF-β can not be made but it is clearly evident that both species normally have TGF-β (active or inactive) within plasma.

Both plasma and peritoneal fluid controls demonstrated an order of variation normally associated with biological variation. Hence this ELISA kit, although intended for detection of human TGF-β detects rabbit TGF-β, accurately and reproducibly (99% homology between species (Kondaiah *et al.*, 1990; Massague, 1990)).

Normal resting levels of TGF-β within plasma and peritoneal fluid appear to be relatively stable. With regard to experimental samples, the large peak in TGF-β at 3 days post injury detected in the plasma sample and elevated levels in peritoneal fluid samples from 5 to 14 days can be seen to have possible biological significance. TGF-β is associated with wound healing and those cell involved in healing, including platelets, fibroblasts, myocytes, astrocytes and epithelial cells. TGF-β is released at wound sites by degranulating platelets and results in cellular chemotaxis, protein matrix synthesis and further secretion of factors which combine with TGF-β to mediate angiogenesis and fibrosis. The limited data obtained so far appears to indicate that TGF-β concentration are elevated during tissue remodelling, that is, collagen synthesis and angiogenesis. In addition, systemic levels of TGF-β are elevated prior to local levels and could be correlated with cellular presence. However the results interpretation is very preliminary and requires substantiating.

Support for elevation of cytokines within peritoneal and wound fluids comes from previous studies. Rong *et al.* (1997) demonstrated elevated levels of TNF-β, GM-CSF, TNF-α and IFN-γ for the first 5 to 7 days post injury and Ford *et al.* (1989) showed high levels of IL-1 and 6, TNF and M-CSF in wound fluid with levels decreasing by the thirteen day. It is hypothesised that these cytokines are secreted at the injury site to promote tissue remodelling (Ford *et al.*, 1989).

Conclusions

i. PSA development may exert an influence on TGF-β levels within plasma and peritoneal fluid sample.
ii. Further experiments are required to authenticate or disprove this hypothesis.

3. 8 Discussion

Scientific interest in PSAs has demonstrated specific trends:

- very few studies investigating PSA formation and maturation
- most studies concentrating on investigating potential PSA agents
- use of a wide variety of experimental models - with different species, different injury types and methods, different levels of severity
- qualitative analysis (usually macroscopic)

When considering these factors it is hardly surprising that to date there is no commercially available preventive agent. In order for PSAs to be prevented detailed knowledge on PSA development is required.

This is the first study researching physiological, structural, cellular (local and systemic) and biochemical mechanisms occurring throughout PSA development, using a definitive experimental model and quantitative analytical procedures (Figure 3.16).

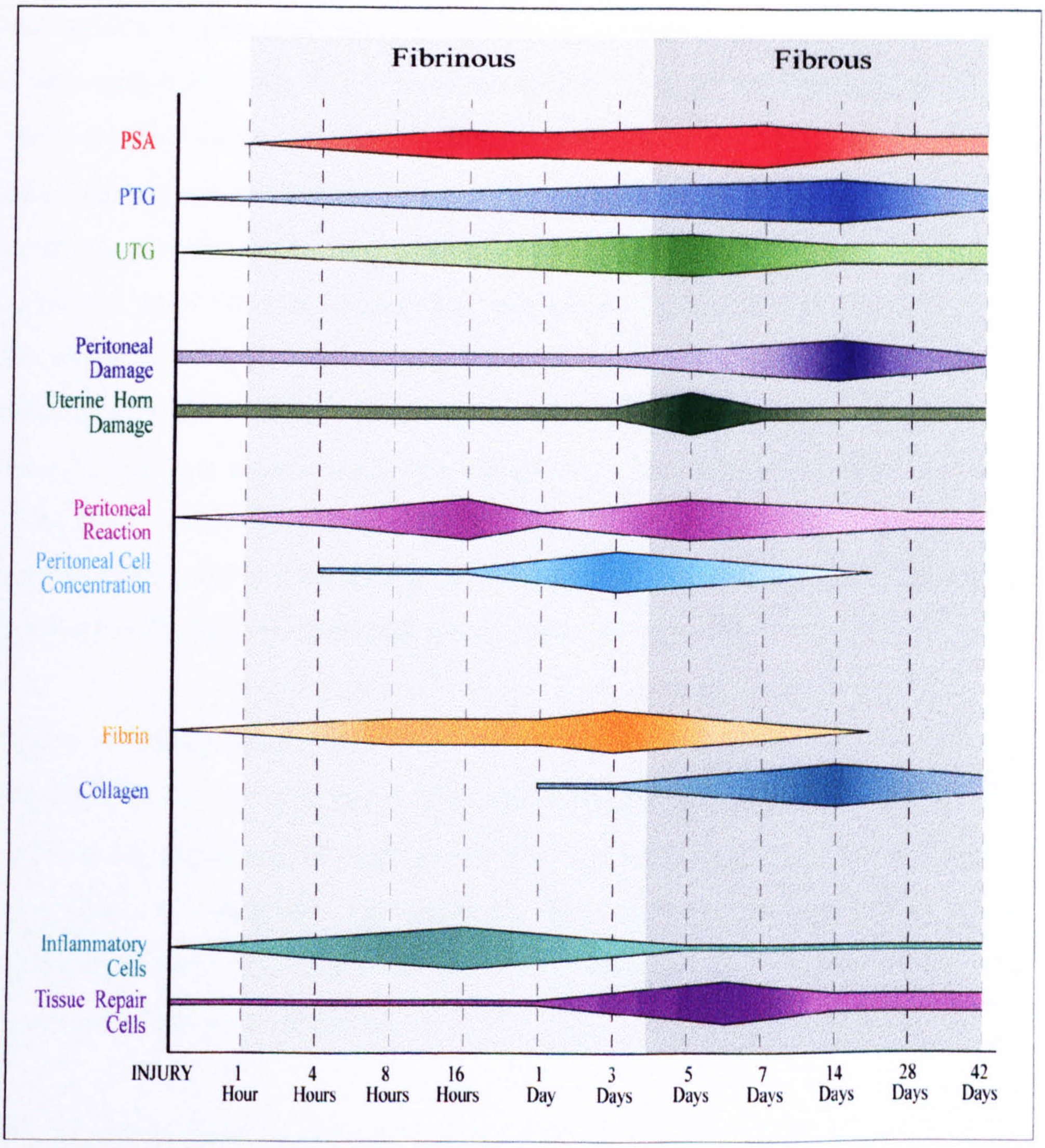


Figure 3.16: Quantitative results for PSA development.

The reaction of peritoneal and uterine horn surfaces to injury was very rapid with fibrin strands seen connecting the two surfaces within 5 minutes and the surfaces adhered *in vivo* within 30 minutes. However it was not until 1 hour that these fibrinous PSAs were tenacious enough to remain intact whilst undergoing tissue resection, slicing and processing. This tenacity was directly related to the structure of the PSA: from 30 seconds to 30 minutes the exudate from and joining the injured surfaces was a blood cell clot primarily composed of exuded blood cells, however, at 1 hour the composition changed with fibrin strands easily visible between the two surfaces. Hence, as soon as fibrin strands were present to provide a framework for a link between surfaces, fibrinous PSAs were formed. By 8 hours following injury, fibrin strands had formed a meshwork and this increased structural strength of the fibrinous PSA had the effect of increasing the incidence of PSAs present, with all cases demonstrating PSAs.

Further development of PSAs occurred during this early phase of formation, with maximum increase per unit time of PSA volume from 4 to 16 hours. This is primarily due to fibrin deposition and corresponds to increased/peak numbers of inflammatory cells, eosinophilic and basophilic cells within the PSA. Hence the infiltration and activity of inflammatory cells was a stimulator for the deposition of further fibrin, probably due to the release of chemical mediators.

However this activity had no effect on the volume of tissue generation present on either the peritoneal wall or uterine horn. It has been generally considered that PSA and tissue generation have the same composition and dynamics, with the only difference being that PSA connects the two surfaces. This study has shown that overall structural and cellular presence throughout development are not significantly different, however it appears that their dynamics are divergent. This is probably a result of the connection of the two surfaces, hence PSAs have cellular and biochemical input from both the peritoneal wall and uterine horn.

In addition to causing lay down of fibrin within PSAs, the infiltration of inflammatory cells seen up to 16 hours post injury also results in a significant increase in peritoneal

reaction and peritoneal cell concentration. Inflammatory cells present within PSAs and, to a lesser extent, tissue generation mount the inflammatory response to limit tissue injury and release chemical mediators which help co-ordinate the inflammatory response and recruit tissue repair cells (fibroblasts and macrophages) into peritoneal tissue to initiate the tissue repair.

From 1 to 3 days following injury, there was a significant decrease in inflammatory cells and significant increase in tissue repair cells. Therefore there was rapid transition between the inflammatory response and tissue repair. This highlights the controlled regulation and co-ordination of PSA development and wound healing.

Peak in the presence of tissue repair cells was seen at 5 days post injury and corresponded to peaks in PSA and tissue generation volumes. Once fibroblasts have entered the wound/PSA they proliferate and synthesise collagen and it was the deposition of collagen which was responsible for the peak in physiological volumes. Hence, by 5 days, fibrinous PSAs have been replaced by established fibrous PSAs.

By 7 to 14 days following injury PSA development was very near completion. Steady decreases in volume were observed due primarily to collagen contraction but also cellular evacuation. Within 2 weeks non adhered tissues were returning to normal. It therefore appears that the formation of PSAs and underlying tissue repair takes approximately the same time as adhesion free healing.

The injury used in this experimental model was more than severe enough for all cases to develop PSAs as a result. However, the overall incidence was between 85-90%. Since all cases from 8 hours to 1 day had PSA present, it is highly probable that all cases did in fact develop fibrinous PSAs. However, some of these fibrinous PSAs were lysed before they formed fibrous PSAs. Support for this hypothesis is found by adhered cases demonstrating greater percentages of fibrin at 5 and 7 days compared to non-adhered cases.

Although systemic cellular changes throughout PSA development were minimal, some effects were observed. Basophilic cell numbers were elevated from 7 to 14 days, monocyte numbers were reduced from 30 seconds to 3 days and a large transient peak in eosinophilic cell numbers was seen at 8 hours. Changes in circulating cells were primarily linked to the inflammatory stage of wound healing and PSA development.

Conclusions

The development of PSAs is a complex, multi-factorial processes, not so much the case of "wound healing gone wrong" but controlled interactions of physiological, structural, cellular and biochemical processes (Figure 3.17). This is the first study to define PSA development into two distinct phases:

i. **PSA modelling**: up to/including 16 hours post injury
   Includes: inflammatory response
   fibrin deposition and maturation

ii. **PSA remodelling**: from 16 hours onwards
   Includes: tissue repair
   collagen deposition and maturation
   chemical mediation by TGF-β

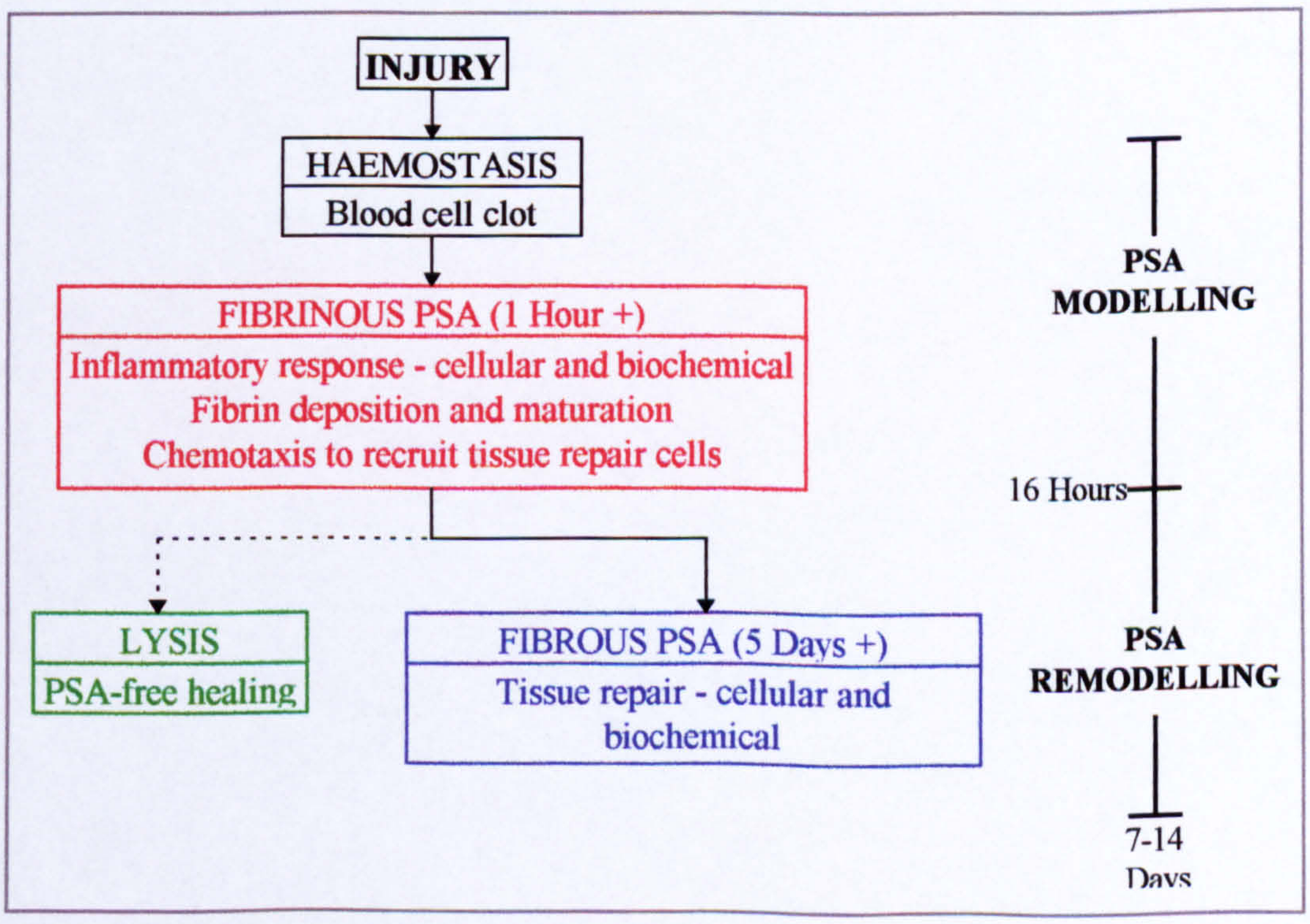


Figure 3.17: Conclusions.

4.0 Prevention of PSAs using Vivostat™ System Human Derived Fibrin Sealant

4.1 Introduction

Problems associated with the presence of PSAs have been recognised since the advent of surgical practices. During this century many potential preventative PSA agents have been tried, often with very little success, and to date no such agent is commercially available. Of all products investigated, physical barriers, especially fibrin sealants have been the most promising.

Even though there are many pre-clinical studies which report a reduction in PSAs with the use of fibrin sealants (Lindenberg & Lauritsen, 1984; Larsson *et al.*, 1986; de Virgilio *et al.*, 1990; Koltai & Gerhard, 1990; Weiss-Fogh *et al.*, 1990; Dulchavsky *et al.*, 1991; Bothin & Hallberg, 1992; Sheppard *et al.*, 1993; De Laco *et al.*, 1994) there are very few clinical studies published (Larsson *et al.*, 1986; Brands *et al.*, 1990; Alnot *et al.*, 1993; Izbicki *et al.*, 1994). There are also studies which report that fibrin sealants do not reduce PSAs (Bilgin *et al.*, 1995) and are associated with potential contamination and antigenicity problems. Currently available fibrin sealants utilise single donor or pooled human fibrinogen, together with exogenous thrombin and usually an anti-fibrinolytic agent such as aprotinin. Using such a system introduces potential risks with regard to viral contamination and the use of bovine products, such as bovine thrombin and aprotinin have risks of bovine spongiform encephalopathy (BSE) and antigenic reactivity (Hughes & Westwood, 1994).

With a view to the above problems ConvaTec™ (BMS company) developed a novel, truly autologous fibrin sealant, derived from a single donor who is intended to be the recipient, with no need for exogenous proteins for fibrinogen cleavage (refer to Section 1.3). Preliminary studies investigating the use of Vivostat™ system human derived fibrin sealant (novel fibrin sealant) as a PSA preventative agent have demonstrated its ability to reduce the formation of PSAs in a rabbit uterine horn abrasion model.

However the mechanisms in which this is achieved are as yet undefined. It has been hypothesised that novel fibrin sealant is acting purely as a physical barrier separating injured surfaces, hence preventing fibrinous connections and, in turn, fibrous PSAs. However since novel fibrin sealant is derived from fresh whole blood it is possible that biochemical mediation of wound healing is also occurring.

Aims of this study

i. Further substantiate the ability of novel fibrin sealant to reduce both incidence and severity of PSAs in a definitive experimental model with quantitative analytical procedures .
ii. Investigate physiological, structural, cellular and biochemical changes occurring during PSA prevention with novel fibrin sealant from 1 hour to 42 days following injury.
iii. Identify possible modes of action for PSA prevention with novel fibrin sealant.

4.2 Materials and Methods

PSAs were induced in a rabbit uterine horn model (refer to Chapter 2). Analogous surgical, termination, sampling and analytical procedures were employed as in the Pathogenesis of PSA study (refer to Chapter 3) for fair and direct comparisons to be made between control and treated tissues. Following the creation of injury sites on the peritoneum and uterine horn positional sutures were tightened to bring the two abraded areas close together but not in contact and fibrin sealant was then applied. Volume of fibrin sealant applied to each experimental site was 0.7ml, applied using the Vivostat™ applicator (150 micron diameter), mid-density spray (26µl/s) (refer to section 2.8.1.3).

The PSA pathogenesis study was completed before this study was commenced and it was found from the former study that tenacious PSAs are first seen at 1 hour post injury. Sampling during this study started at this time point and at 4, 8, 16 hours, 1, 3, 5, 7, 14, 28 and 42 days following injury.

Resected tissues were fixed and transverse slices were cut in a uniform random manner (refer to Chapter 2). Odd numbered slices were processed to paraffin wax for volumetric analysis and even numbered slices were processed to resin for number density analysis (refer to Chapter 2).

4.3 Macroscopical Analysis

During the course of this study there were no mortalities and no animal demonstrated morbidity associated with the surgical procedure.

All cases had mild to moderate PSAs associated with laparotomy, that is, PSAs involving the peritoneal suture line and underlying tissues, either the fat pad over the bladder and/or the bladder itself. Several cases in addition had mild to moderate PSAs involving the distal suture(s) of experimental sites with the fat pad over the bladder and/or the bladder itself. All of these PSAs were blunt dissected with relative ease and did not interfere with experimental sites.

Macroscopically it was not possible to assess PSA presence at experimental sites in cases up to 3 days. In most of these cases the two abraded areas were joined together, but whether this was due to fibrin sealant or PSAs was undetermined at macroscopic level. All cases from 3 days onwards were easily identifiable as not adhered (Figures 4.1 and 4.2) except for one 28 days case which was partially adhered at both left and right hand side experimental sites. Non-adhered injury areas were separate and clean, and appeared to be healing without problems.

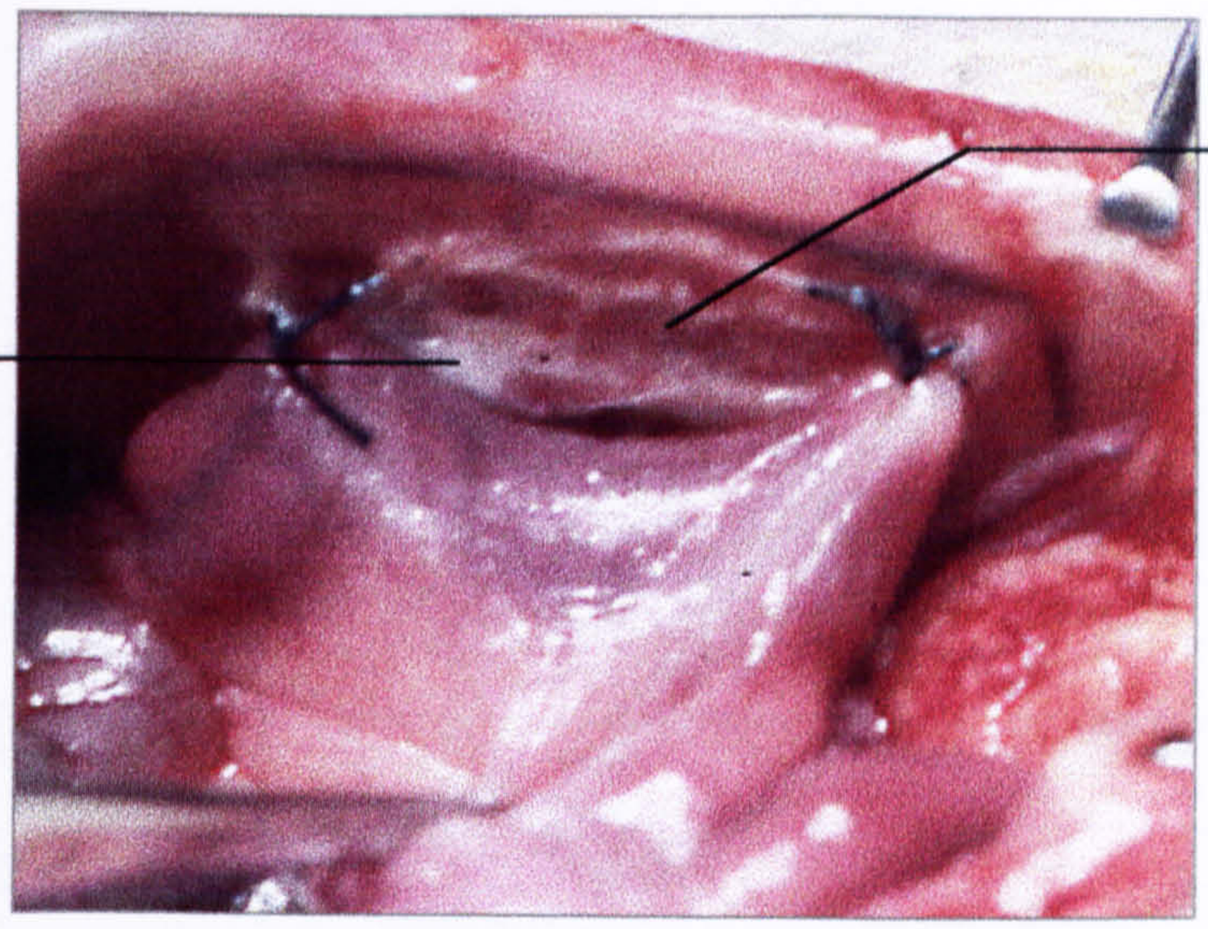


Figure 4.1: Macroscopic appearance of novel fibrin sealant treated injury site at 3 days post injury.

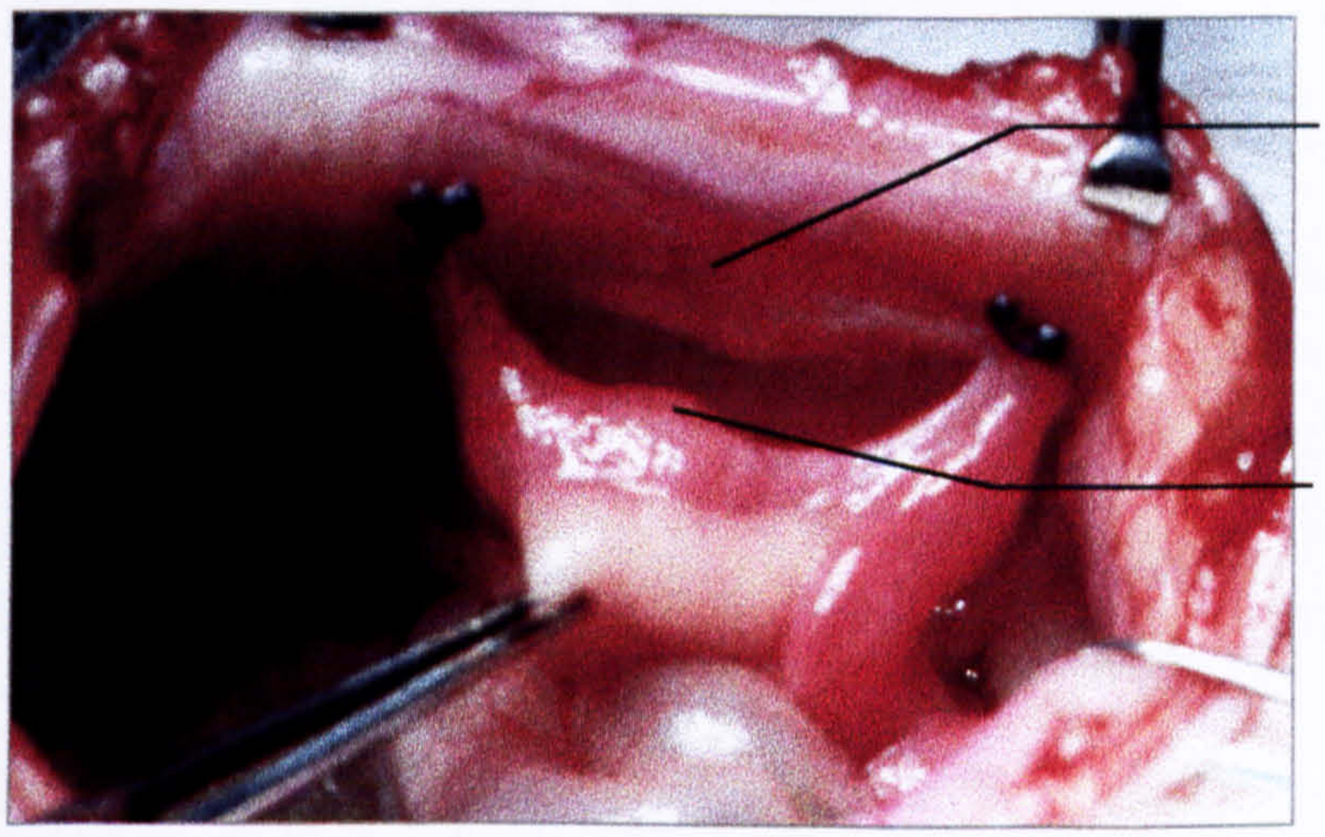


Figure 4.2: Macroscopic appearance of novel fibrin sealant treated injury site at 14 days post injury.

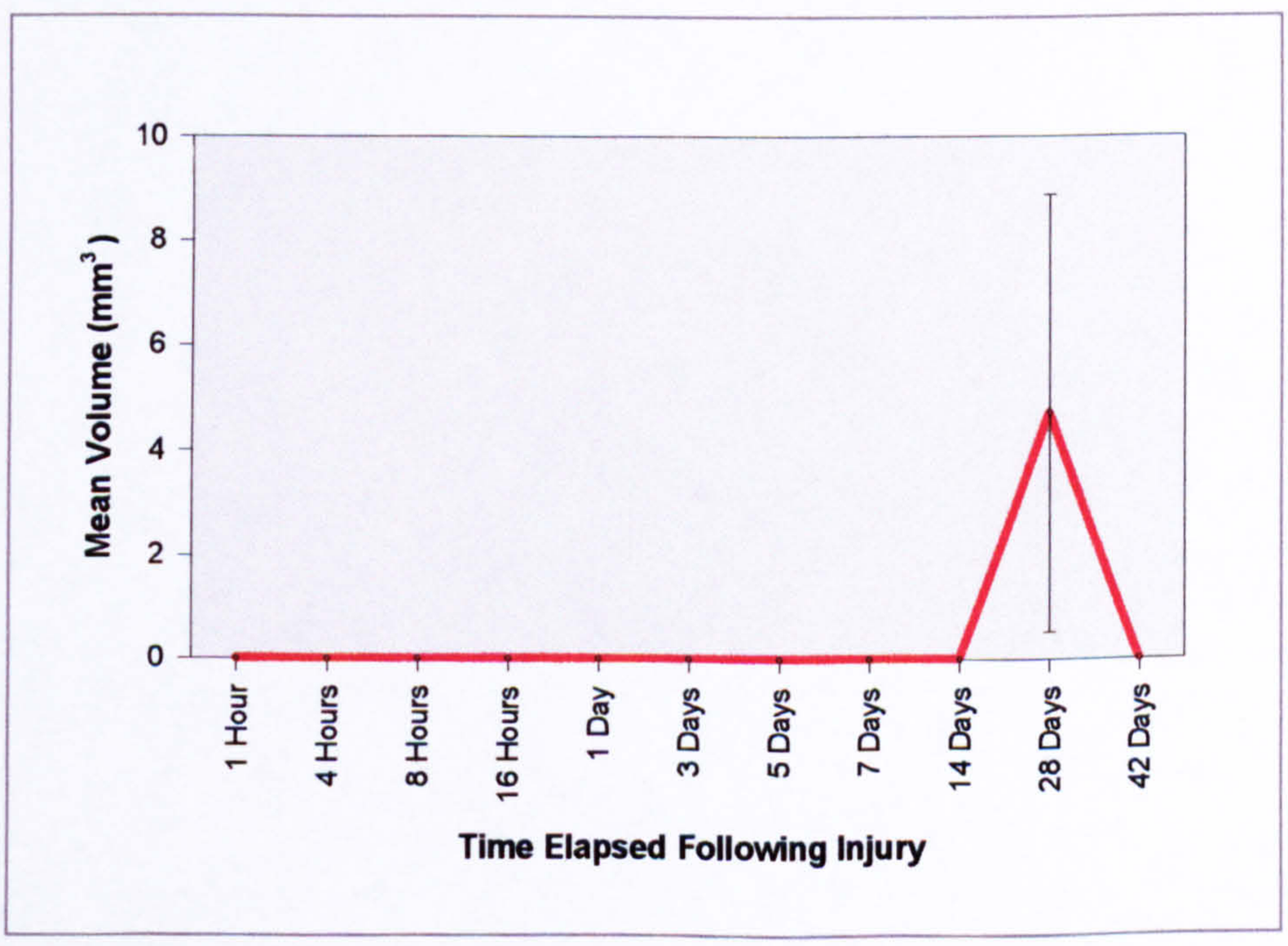
4.4 Volumetric Analysis of Tissue Zones

In order to determine the mechanisms and timing of tissue repair with novel fibrin sealant physiological changes were quantified in an analogous method to PSA development. Volumes of PSA, tissue generated by and damage of the peritoneal wall and uterine horn, peritoneal reaction, peritoneal cell concentration, uterine horn haemorrhage, fibrin sealant remaining and fibrin sealant associated cell concentration were determined from 1 hour to 42 days.

Fixed resected tissues were uniform randomly sliced, with odd numbered slices were processed to paraffin wax. A 5μm section was cut from each block and stained with haematoxylin and eosin. Absolute volume of each tissue zone was then determined using the stereological technique of Cavalieri's principle (refer to Chapter 2).

4.4.1 Results

Microscopically PSAs were only evident in one animal, a 28 day case (Graph 4.1; Appendix IV Table 4.1). Both experimental sites were partially adhered, with 33.80mm^3 of PSA present at the LHS experimental site and 3.91mm^3 present at the RHS site. However there was no statistically significant difference ($P>0.05$) between the 28 day group compared to other sample groups (Appendix IV Table 4.2). Overall the use of novel fibrin sealant produced a 98% reduction of PSAs in this study (Figure 4.3 and 4.4).



Graph 4.1: Mean PSA volumes together with standard error of the mean (SEM).

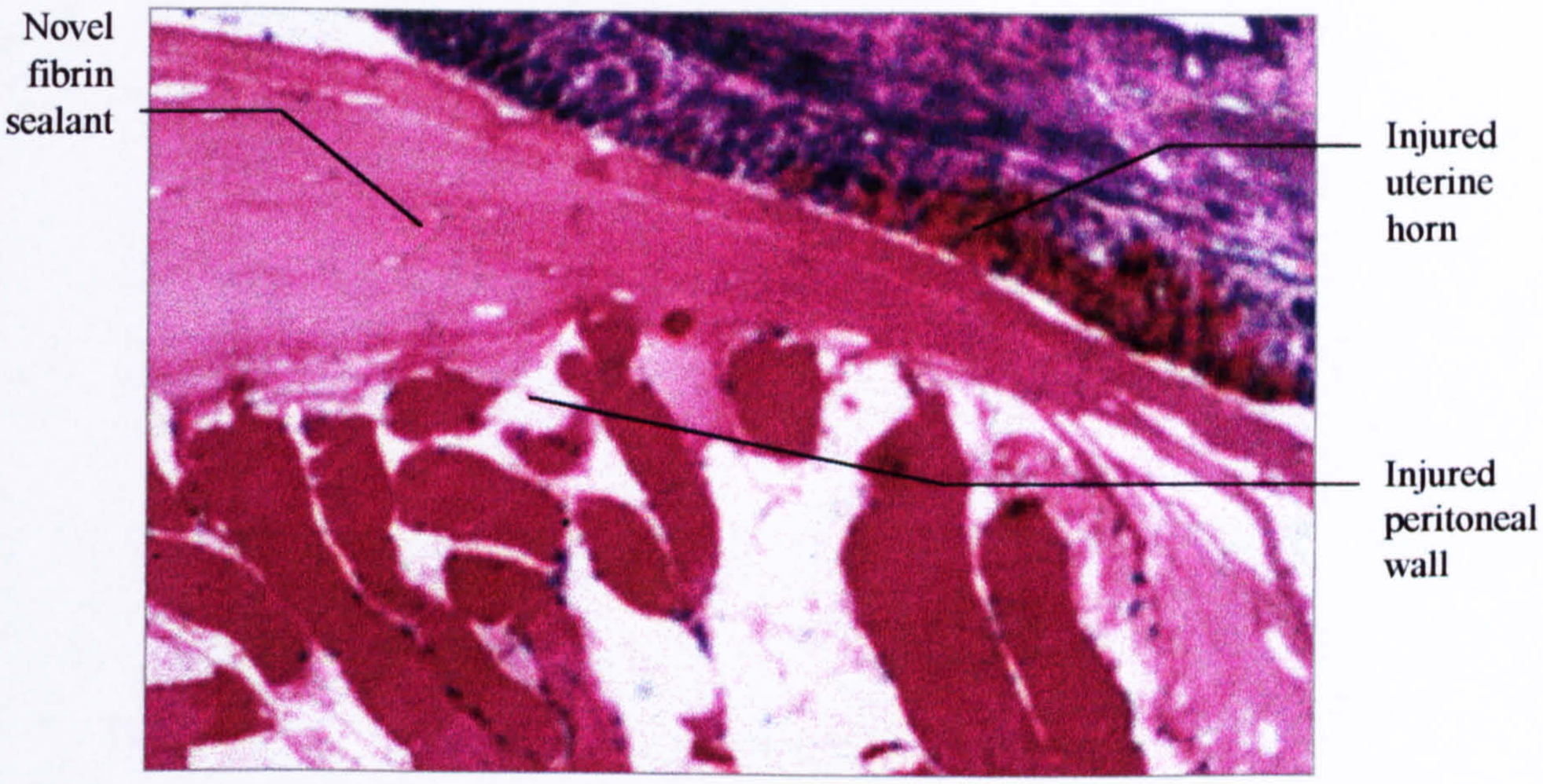


Figure 4.3: Tissue generation at 1 hour post injury.
(M_o x10)

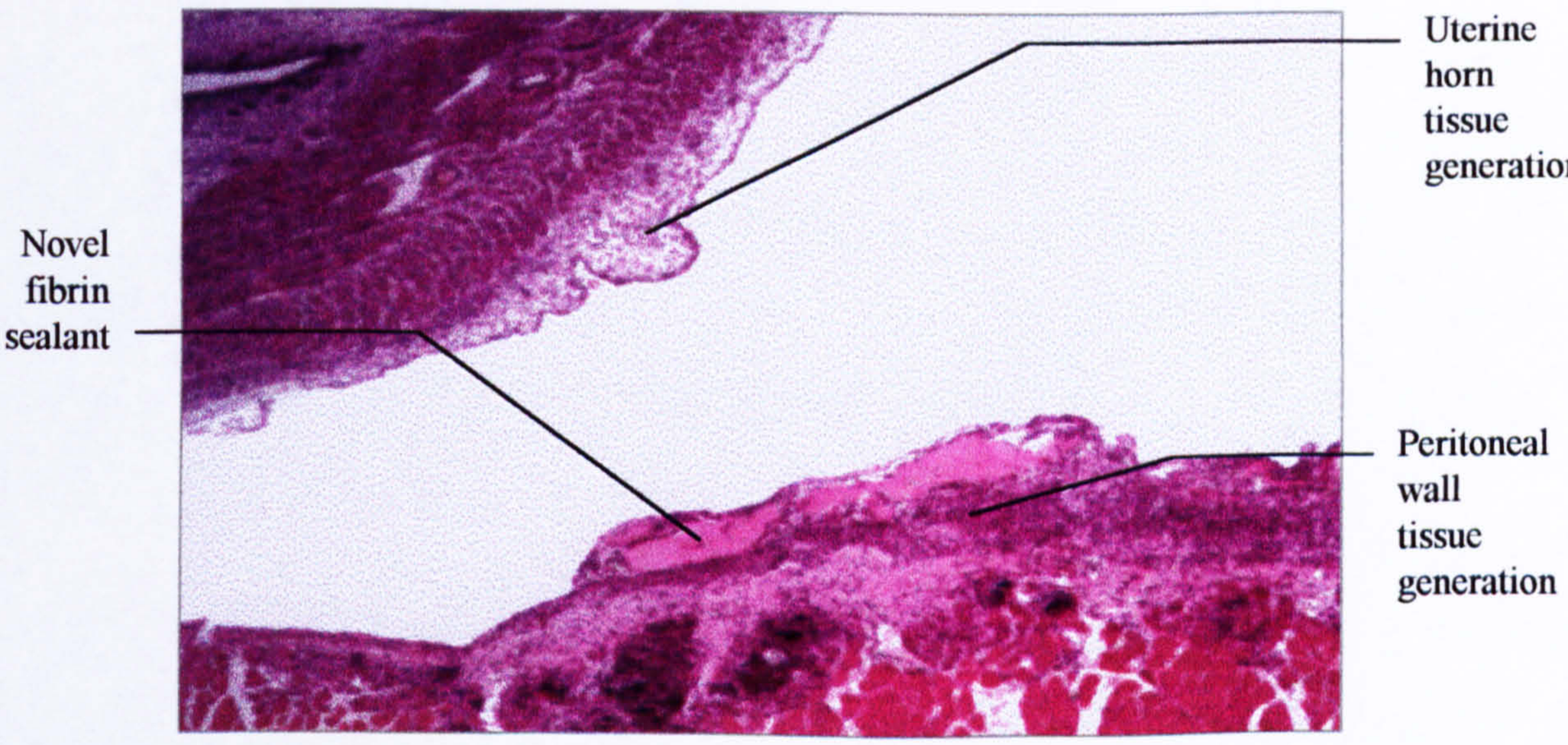


Figure 4.4: Tissue generation at 7 days post injury.
(M_o x4)

Peritoneal tissue generation (PTG) was present at all sample time points (Graph 4.2; Appendix IV Table 4.1), with a trend for mean volume to increase up to 7 days, with significant increases at 4 hours ($P<0.05$), 1 day ($P<0.001$) and 5 days ($P<0.05$) (Appendix IV Table 4.2; Figure 4.5). From 7 days onwards a trend for mean volume to decrease was seen although there were no significant differences ($P>0.05$) from time point to time point.

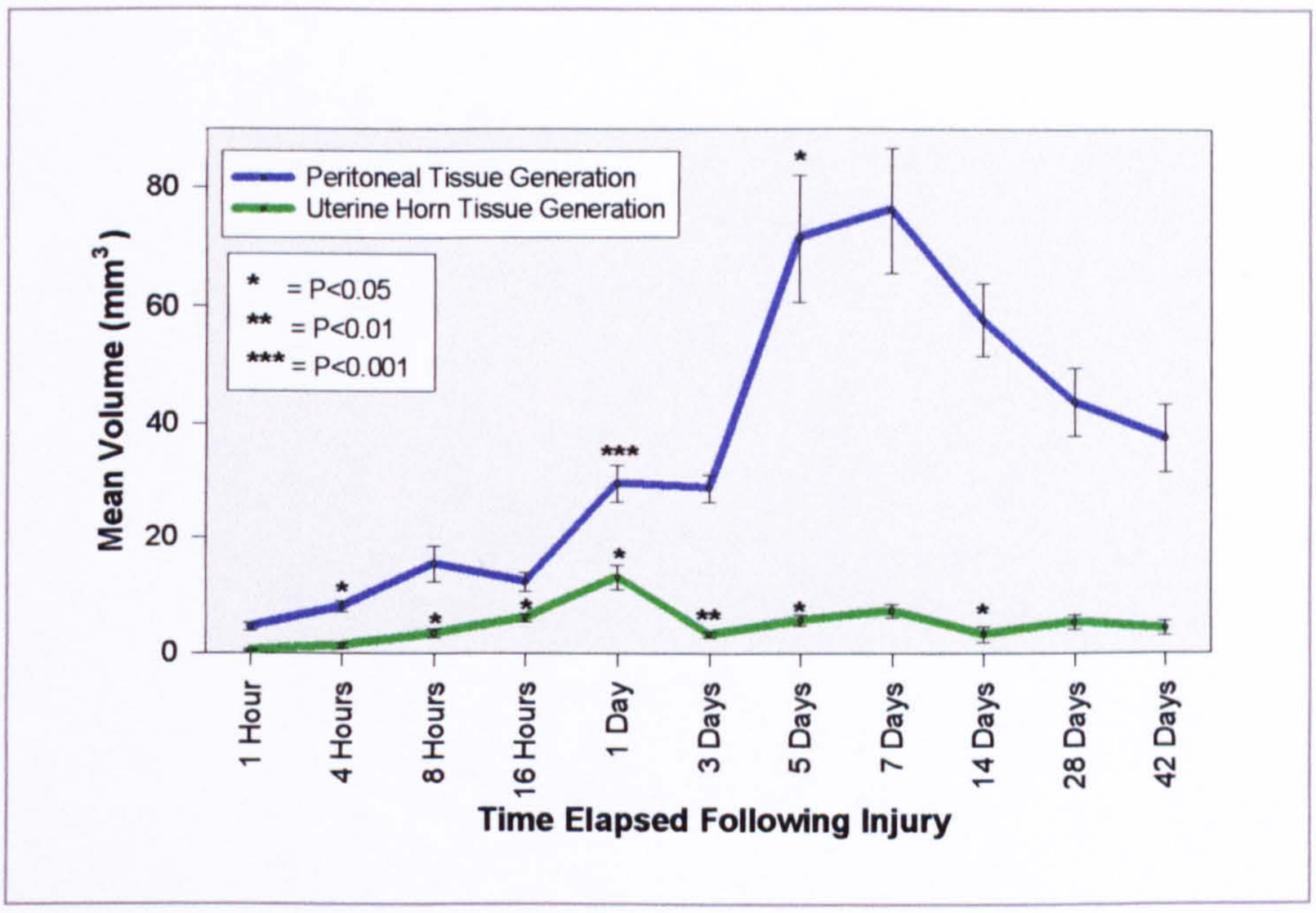
Uterine horn tissue generation (UTG) was evident at our first time point of 1 hour and all

subsequent time points (Graph 4.2; Appendix IV Table 4.1). Initially there was an increase in mean volume, peaking at 1 day, with significant increases ($P<0.05$) seen at 8 and 16 hours and 1 day. At 3 days there was a significant decrease in volume ($P<0.01$), with another significant decrease ($P<0.05$) at 14 days (Appendix IV Table 4.2). Mean volume there after remained at a constant low level (Figure 4.6).

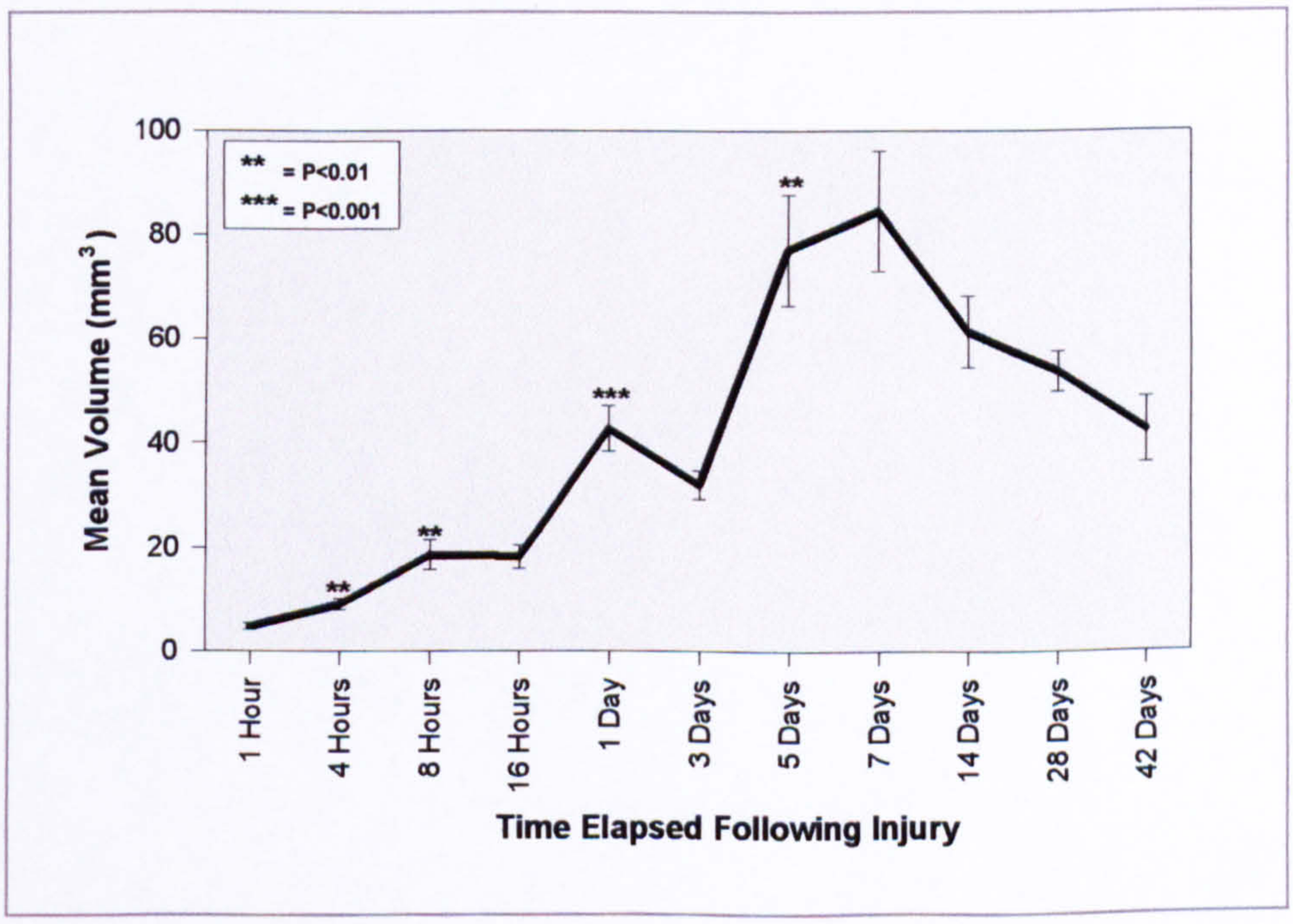
Mean total tissue generation (TTG) showed an increase in volume up to 7 days post injury (Graph 4.3; Appendix IV Table 4.1), with significant increases seen at 4 hours ($P<0.01$), 8 hours ($P<0.01$), 1 day ($P<0.001$) and 5 days ($P<0.01$) (Appendix IV Table 4.2). From 7 days onwards mean volume decreased with each time point although no significant differences were seen.

Mean volume for peritoneal damage showed a trend to decrease from 1 to 16 hours although not significantly ($P>0.05$) (Graph 4.4; Appendix IV Tables 4.1 and 4.2). From 16 hours to 5 days mean volume of peritoneal damage increased, peaking at 5 days, with significant increase ($P<0.05$) at 1 day. From 5 days onwards mean volume decreased steadily until the end of the study, a significant decrease ($P<0.001$) was seen at 14 days post injury (Figure 4.5).

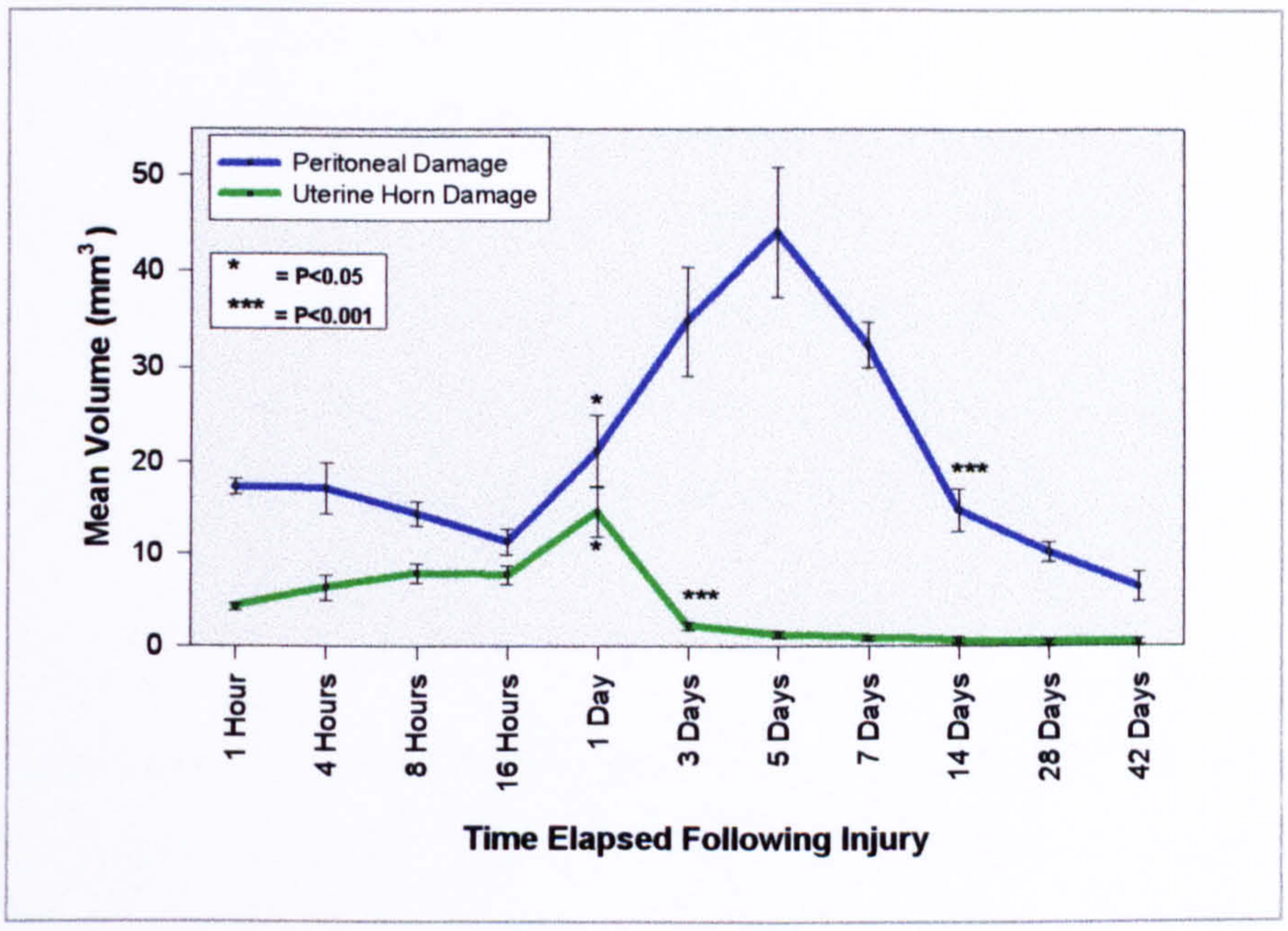
Uterine horn damage increased from 1 hour to 1 day, with a significant increase ($P<0.05$) at 1 day following injury (Graph 4.4; Appendix IV Tables 4.1 and 4.2). There was a significant decrease ($P<0.001$) in mean volume at 3 days and mean volumes for damage decreasing gradually there after (Figure 4.6).



Graph 4.2: Mean tissue generation volumes
together with SEMs and statistical differences.



Graph 4.3: Mean TTG volumes
together with SEMs and statistical differences.



Graph 4.4: Mean damage volumes
together with SEMs and statistical differences.

Figure 4.5: Peritoneal damage and tissue generation at 14 days post injury.
(M_o x4)

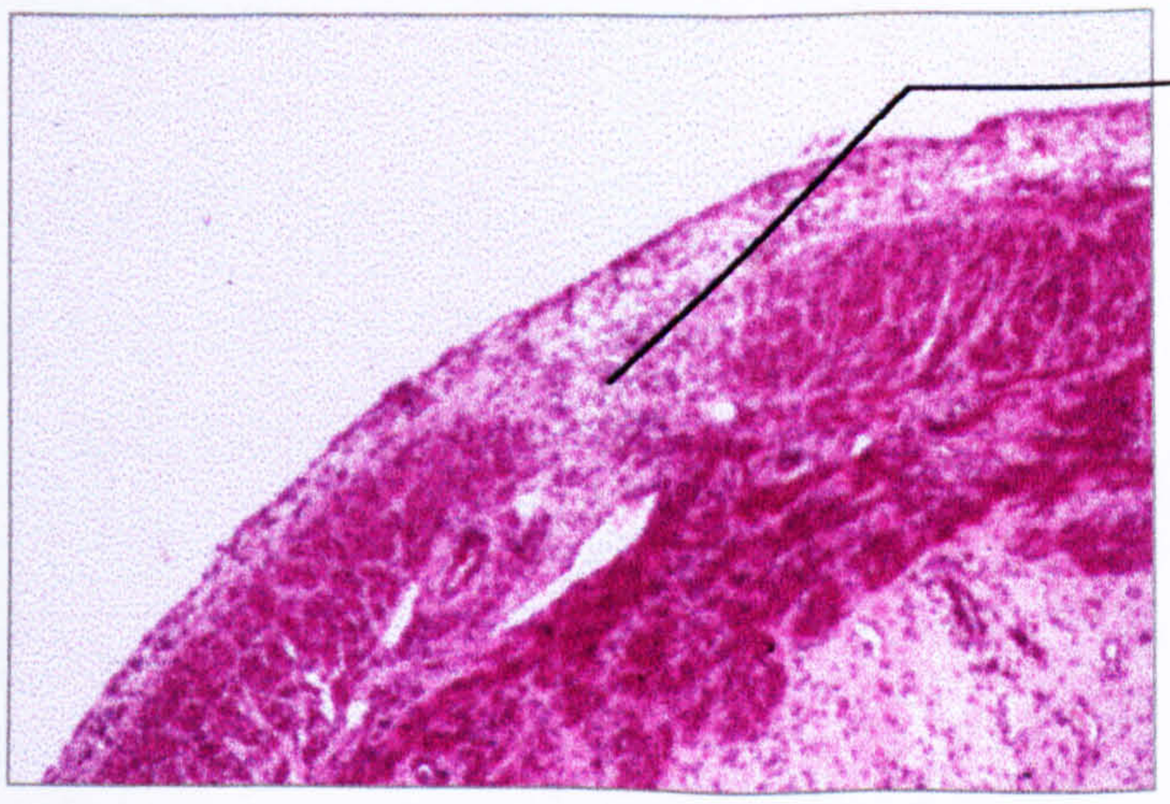


Figure 4.6: Uterine horn damage and tissue generation at 5 days post injury.
(M_o x10)

Peritoneal reaction was present throughout the whole of our study (Graph 4.5; Appendix IV Table 4.1). Mean volume increased significantly at 4 hours ($P<0.0001$), 8 hours ($P<0.01$) and 1 day ($P<0.01$) (Appendix IV Table 4.2). A significant decrease was seen at 3 day post injury ($P<0.01$) followed by a significant increase at 5 days ($P<0.01$). From 5 to 7 days there was little change in mean volume. However at 14 days following injury a significant decrease was seen ($P<0.0001$), with volume remaining constant until the end of the study.

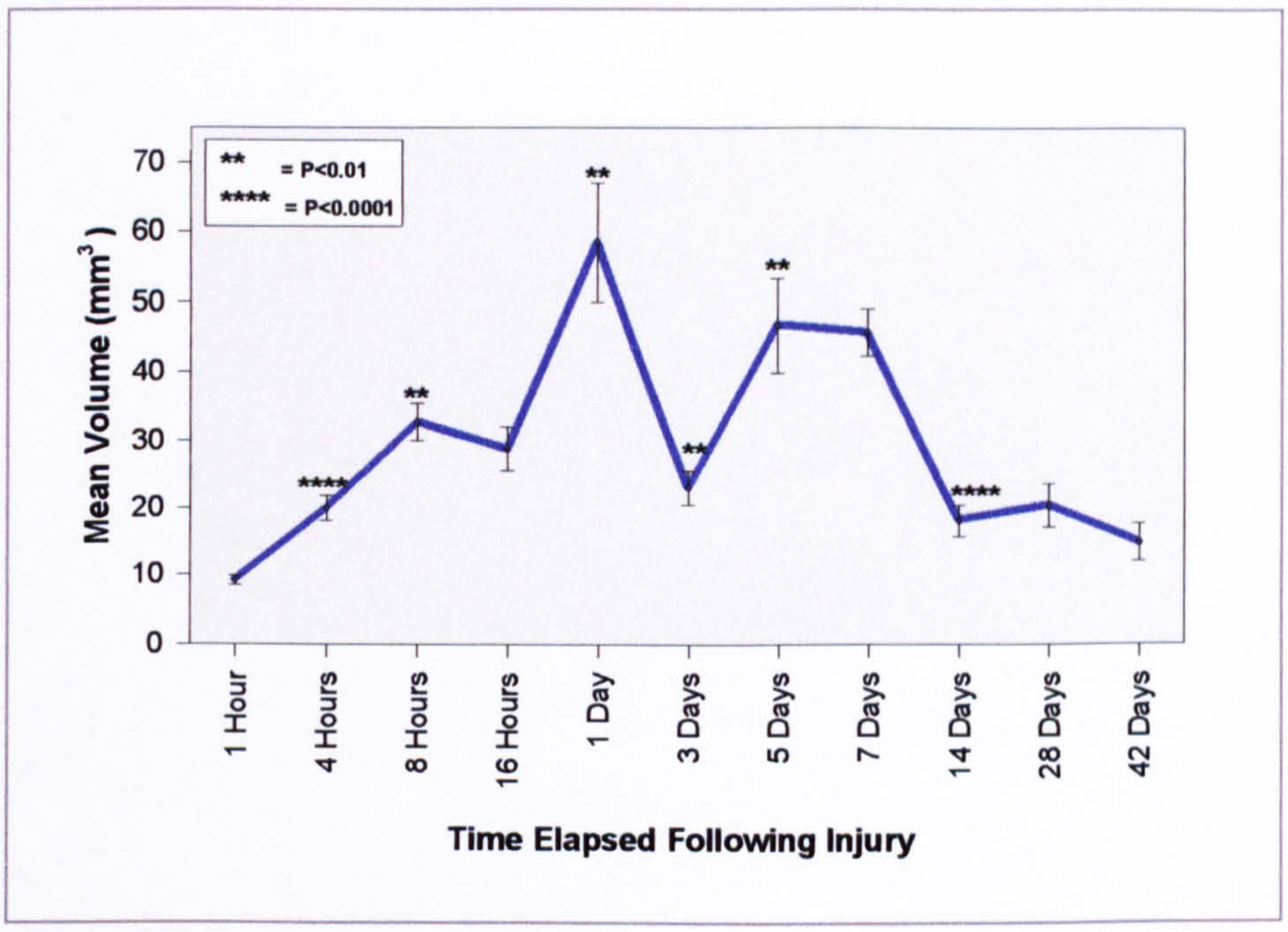
Very little peritoneal cell concentration was evident throughout this study (Graph 4.6; Appendix IV Table 4.1). Up to 3 days post injury there were no detectable levels. At 5 days there was a peak in mean volume although this was only transient as volume decreased after this, with a significant decrease ($P<0.01$) at 14 days (Appendix IV Table 4.2). No peritoneal cell concentration was seen at either 28 or 42 days post injury.

Uterine horn haemorrhage was present only up to 1 day following injury, with slight peaks in mean volume seen at 1 and 16 hours although not significantly ($P>0.05$) (Graph 4.7; Appendix IV Tables 4.1 and 4.2).

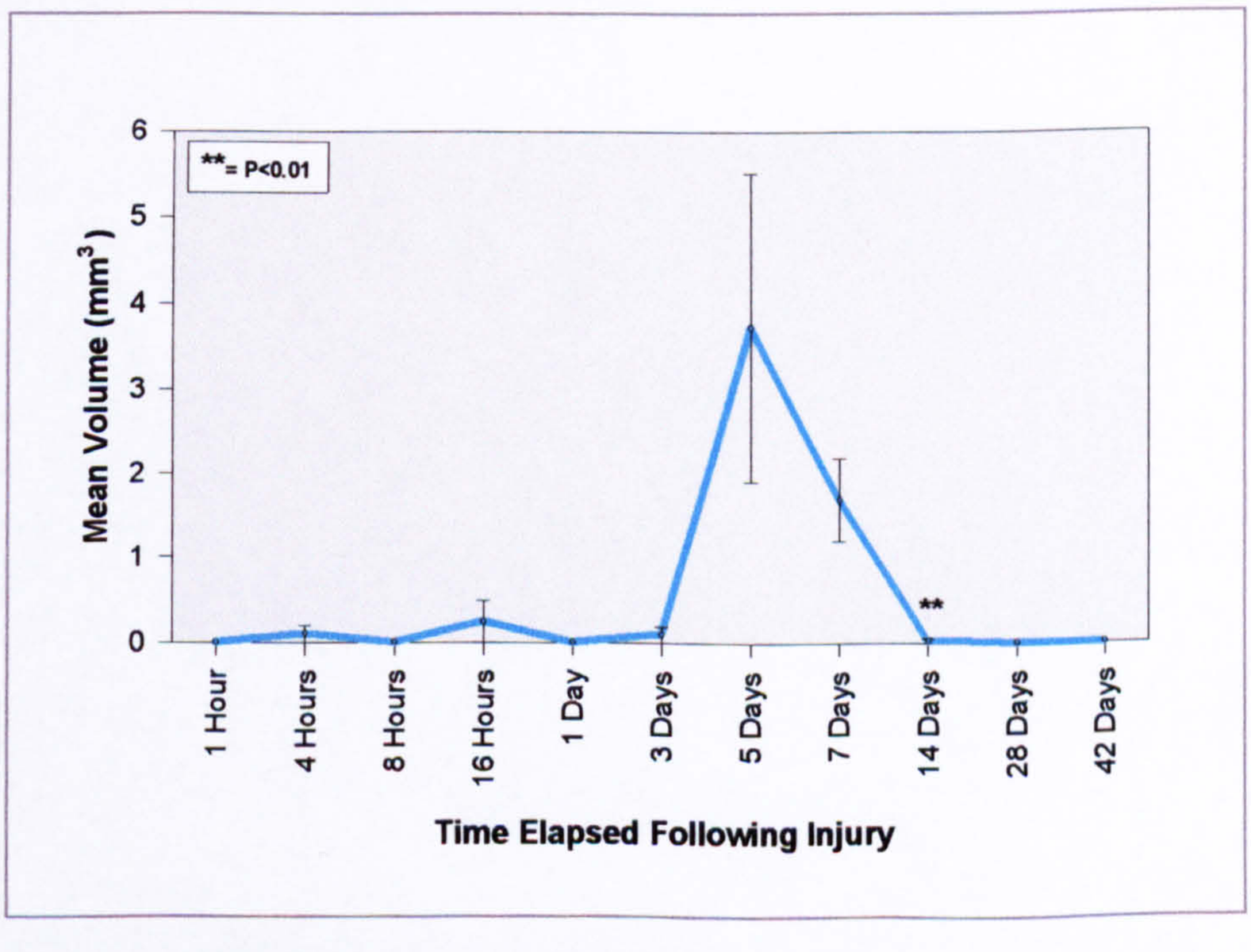
Human derived fibrin sealant was present at all injury time points except 42 days (Graph 4.8; Appendix IV Table 4.1). There was a trend for mean volume to decrease between 1 and 16 hours, a significant decrease ($P<0.001$) at 4 hours. A relatively constant mean volume was evident between 16 hours and 5 days followed by a significant decrease was seen at 7 ($P<0.01$) and 14 days ($P<0.001$) post injury (Appendix IV Table 4.2).

Fibrin sealant associated cell concentration was first present at 3 days following injury, increasing significantly (1.27 fold)($P<0.001$) to peak at 5 days. At 7 days there was a trend for mean volume to decrease with a significant decrease ($P<0.001$) at 14 days. Mean volume remained low until 28 days and cell concentration absent at 42 days post injury (Graph 4.8).

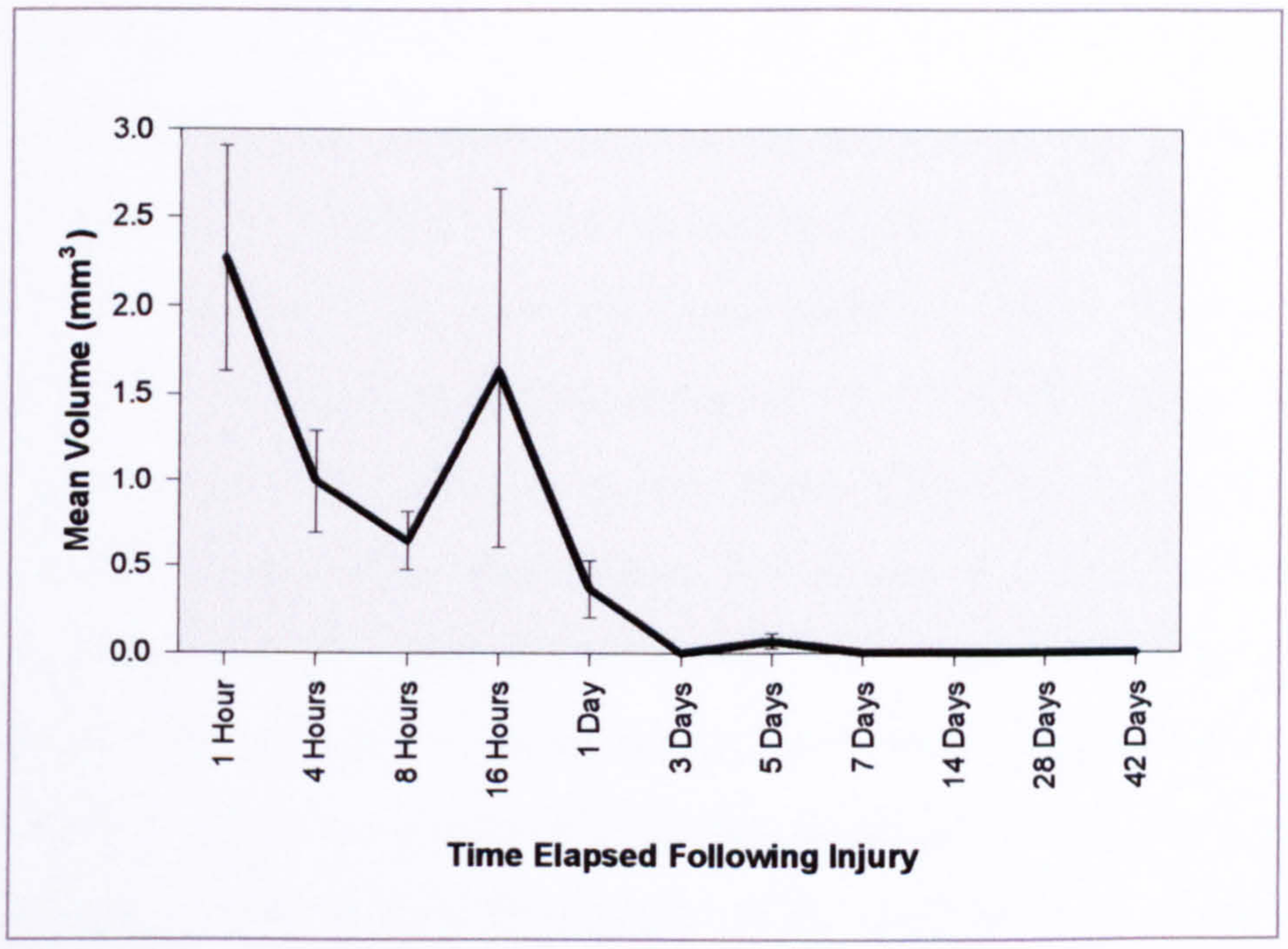
The coefficient of error for Cavalieri's analysis in this study was 9%.



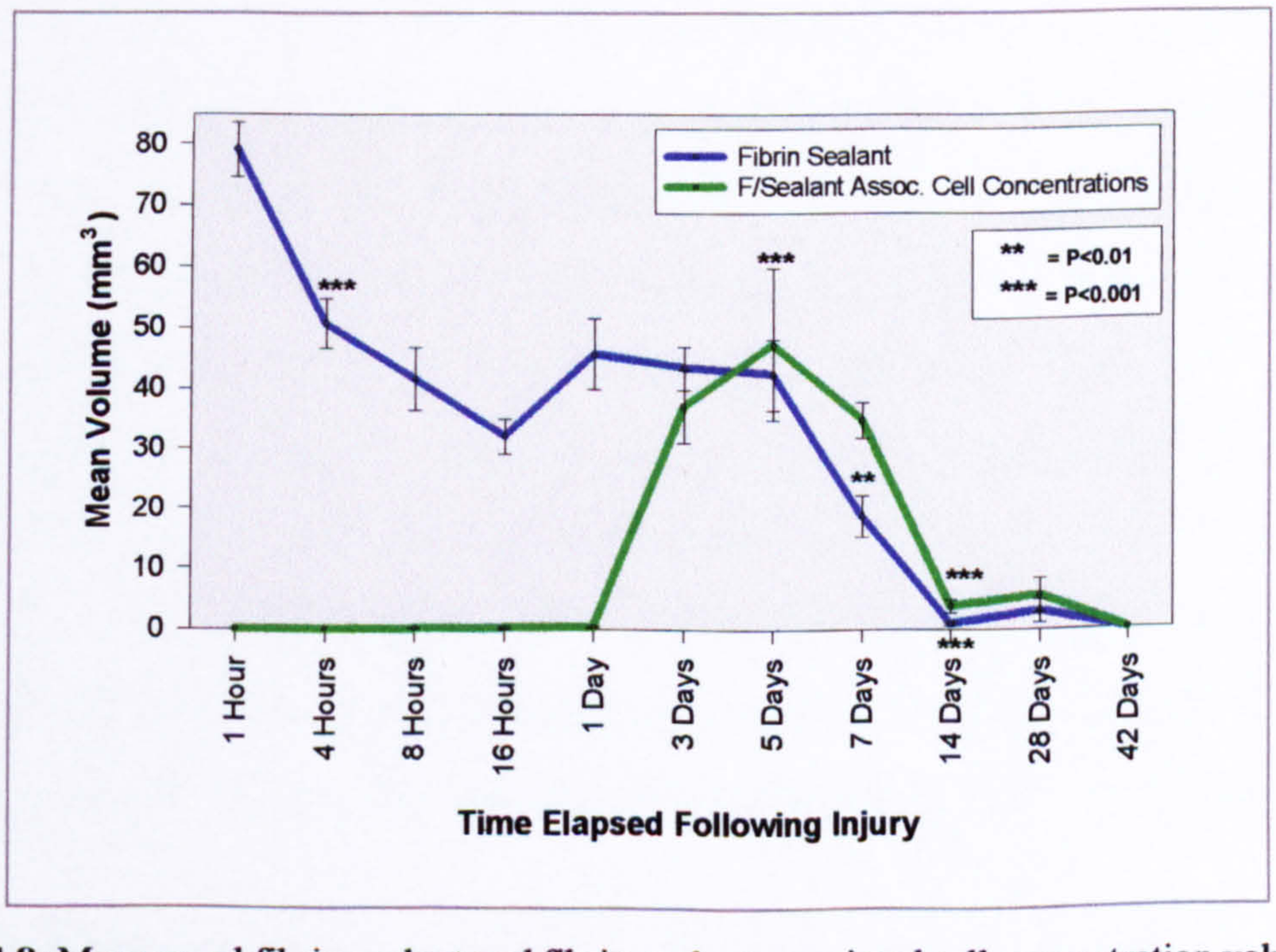
Graph 4.5: Mean peritoneal reaction volumes
together with SEMs and statistical differences.



Graph 4.6: Mean peritoneal cell concentration volumes
together with SEMs and statistical differences.



Graph 4.7: Mean uterine horn haemorrhage volumes together with SEMs.



Graph 4.8: Mean novel fibrin sealant and fibrin sealant associated cell concentration volumes together with SEMs and statistical differences.

4.4.2 Discussion

Novel fibrin sealant has the ability to significantly prevent PSAs in a definitive rabbit uterine horn abrasion model with 98% prevention in this study. Only one case out of 44 showed PSA development. During surgery it was noted that this case received a severe injury to the LHS peritoneal wall and application of novel fibrin sealant was poor due to technical problems with the application system. These factors most probably contributed to PSA formation at the LHS experimental site in this animal. However the RHS experimental site also had a small PSA between the two abraded sites. Therefore it appears that this animal had increased fibrinogenic capacity or impaired fibrinolytic capacity, making it more susceptible to PSA development. Analysis of peritoneal fluid and plasma samples collected from this animal will hopefully substantiate this hypothesis.

Tissue generated in response to abrasion injury demonstrated a similar pattern on both the peritoneum and uterine horn: constant/slowly increasing volumes during the first 16 hours, followed by a significant increase ($P<0.05$) to peak at 5 to 7 days for the peritoneal wall and 1 day for the uterine horn and then decreasing volumes thereafter. From previous studies (Johnson & Whitting, 1962; Eskeland, 1966.1; Eskeland, 1966.2; Raftery, 1973.L; Raftery, 1973.E; Ryan *et al.*, 1973) (refer to section 1.1.6.3.2 of this thesis) we know that during adhesion-free healing, tissue generated in response to injury is composed of fibrin during the first 3 days, with fibrin lost thereafter and collagen taking its place. An analogous situation was found for PSA composition by Milligan & Raftery (1973) (refer to section 1.1.6.5.1 of this thesis). During the first 16 hours of healing with novel fibrin sealant there was a slow rate of tissue generation deposition on the injury surface. This is most probably due to the presence of the sealant. After this time fibrin is laid down at an increased rate with collagen deposition following thereafter.

Volumes of total tissue generation showed two peaks, the first at 1 day corresponding to a peak in volume of fibrinous tissue generation (fibrin predominant) and a second peak at 5 to 7 days, corresponding to a peak in fibrous tissue generation (collagen predominant). Interestingly although these two trends can be seen in both PTG and UTG, the peak in fibrinous tissue generation corresponds primarily to maximum UTG volume and the

fibrous peak to maximum PTG volume. It would appear that uterine horn generates its maximum repair tissue 4 to 6 days earlier than the peritoneum and undergoes noticeable fibrin loss, either by fibrinolysis and/or phagocytosis. Reasons for this could be: (i) the greater vasculature of the uterine horn; (ii) fibrin sealant remaining attached to the peritoneal wall in preference to the uterine horn leaving the uterine horn available for fibrinolysis at an earlier stage; or (iii) the uterine horn sustained a lower level of underlying tissue damage from the abrasion procedure. The latter hypothesis is the most probable and is explained below.

Less repair tissue volume was generated by the uterine horn in response to injury compared to the peritoneal wall and this has been a common feature in all studies carried out in this Department. It is well recognised that there is a direct relationship between the amount of damage and tissue generated from that damage (Ellis, 1962; Belzer, 1967; Chapter 3 of this thesis). It is hypothesised that lower volumes of UTG compared to PTG are due to the fact that, although going through the same injury process, the uterine horn received a lower extent of underlying damage compared to the peritoneal wall. This is probably due to the differences in structure between these two organs, with the peritoneum "easier to injure". This highlights the importance of "non-visible" damage of tissues during surgical procedures and the formation of fibrotic tissue and especially PSAs.

Mean damage for uterine horn was significantly less ($P<0.05$) compared to the peritoneal wall for all sample time points. In previous studies in our Department we found that, initially, the level of damage for the two tissues was not statistically different, but after 3 days there was a difference (refer to Chapter 3). This was due to both tissues receiving the same level of initial damage, with the peritoneal wall sustaining a greater level of underlying damage. Since the identical injury technique was used in this study it appears that novel fibrin sealant has some damage limitation effect on the uterine horn very quickly after injury induction. This could be due to damaged tissue being enclosed by fibrin sealant and not in direct contact with the peritoneal environment.

With regard to the pattern for peritoneal and uterine horn damage similar trends were evident: a constant volume of damage for the first 16 hours corresponding to the original volume of damage inflicted via the abrasion injury, followed by an increase due to tissue necrosis of damaged underlying and surrounding tissue with its subsequent removal by phagocytes following the normal procession of wound healing. Following the peak in damage, volumes decreased for the remainder of the study, with volumes lower by the end of the study then at 1 hour post injury. This indicates that wound healing has occurred successfully and damage tissue has been replaced with new repair tissue. In addition to actual damage seen within the uterine horn there were also small amounts of haemorrhage present from 1 hour to 1 day following injury.

A reaction to injury was mounted very quickly within peritoneal tissue with detectable levels seen after 1 hour. Peritoneal reaction showed a multi-peak response pattern, peaking at 8 hours, 1 and 5 days. This follows a familiar pattern for cellular activity during wound healing. Following injury an inflammatory response is mounted. Neutrophils are recruited to the injury sites within several hours, being one of the predominant cell types between 1 and 3 days post injury (Johnson & Whitting, 1962; Hubbard *et al.*, 1967; Ryan *et al.*, 1973; Raftery, 1973 E). Neutrophils release chemotactic mediators, attracting other phagocytic cells, such as macrophages. When present at the injury site these phagocytic cells clear damaged/necrotic tissues in order for wound healing to process. Within the first 3 to 5 days much chemical and cellular activity is occurring within injured tissues and it is this activity which we are detecting as peritoneal reaction. Cellular concentrations within peritoneal tissue also correlate with that for reaction, with cellular activity being at its most intense at 5 days post injury.

At our first sample time point of 1 hour, approximately 11% of the novel fibrin sealant sprayed was present at the injury site. This very large decrease in volume is due to: (i) loss of sealant through the aerosol process (approximately 15%); (ii) sealant sprayed not reaching or adhering to the injury site (approximately 35%); (iii) loss of liquid from the sealant through compression and processing (approximately 40%). Thus the volume of sealant present at 1 hour is more representative of the actual volume of fibrin present

within the sealant at the site of injury. A further decrease was seen at 4 hours, however, even with this loss of volume, novel fibrin sealant was still *in situ*, remaining between and joining the two injured surfaces. From 4 hours to 5 days post injury there were no significant differences ($P>0.05$) in mean volume, although subtle changes are occurring, with the sealant gradually not joining the two injuries surfaces. A rapid lysis of sealant occurred between 7 and 14 days, with minimal levels present at 14 and 28 days. All traces of fibrin sealant were absent at our final sample time point of 42 days. Therefore once *in vivo* novel fibrin sealant remains relatively unchanged during the first 5 days post injury/surgery, that is during PSA and tissue generation modelling and remodelling, with biodegradation thereafter.

In association with novel fibrin sealant a reactive cell concentration was seen surrounding the sealant and in adjacent tissues from 3 days onwards, peaking at 5 days. This peak was only transient, with mean volume decreasing rapidly to 28 days, with no detectable levels at 42 days. As seen in previous studies, this is a cellular reaction mounted in response to the presence of foreign protein (human novel fibrin sealant) within a non-human experimental model. This reaction, however, appears to have little to no detrimental effects on the outcome of the wound healing of these animals or on PSA prevention.

Conclusions

i. Novel fibrin sealant totally prevented PSAs in 98% of cases in this experimental study.
ii. The normal progression of wound healing was unaffected by the use of novel fibrin sealant.
iii. Limitation of subsequent damage in underlying and surrounding tissues to the injury site was demonstrated by novel fibrin sealant.
iv. Once *in vivo* novel fibrin sealant maintained at a relatively constant volume and position within the peritoneal cavity for 5 days post injury, with gradual lysis thereafter.

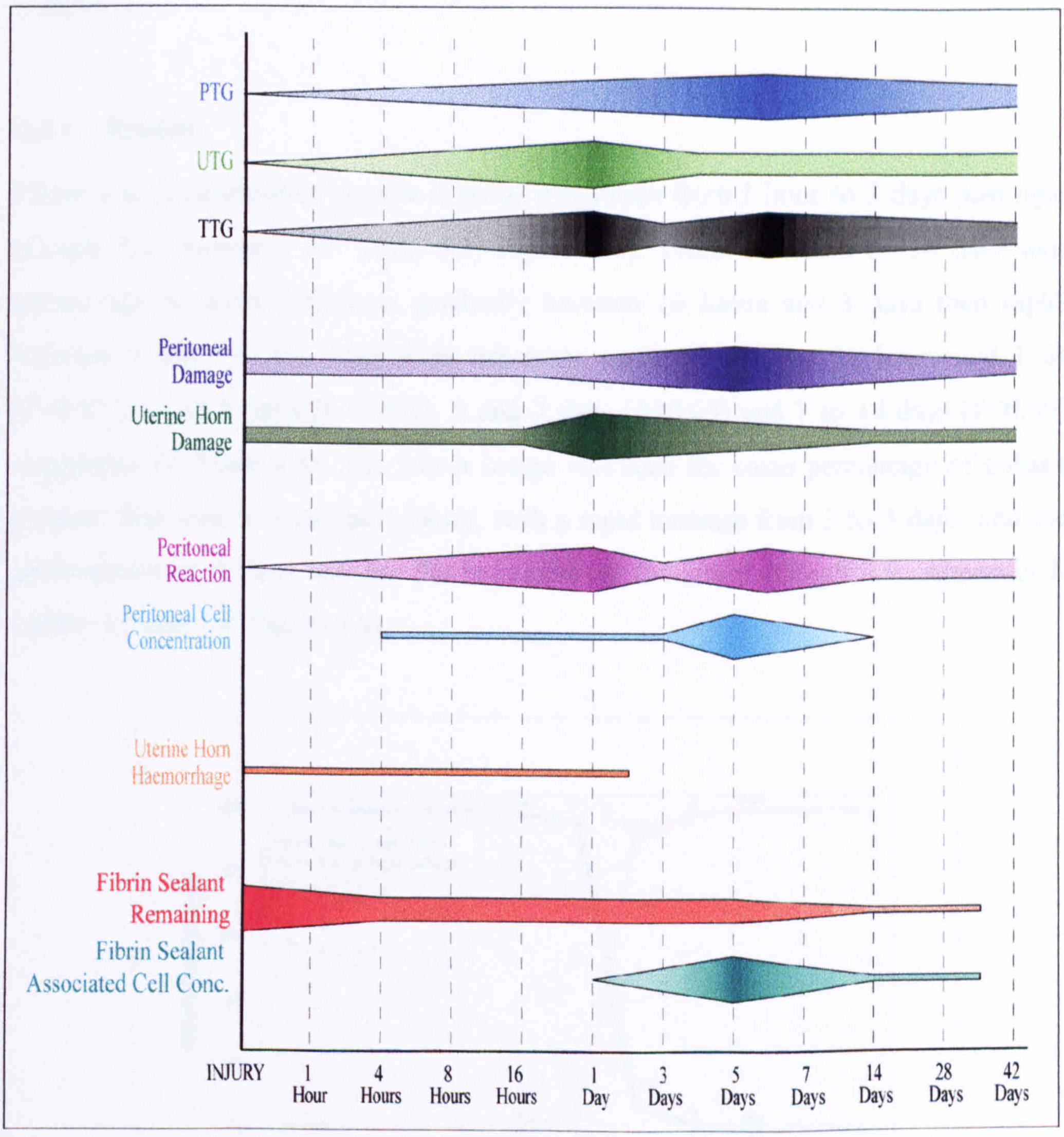


Figure 4.7: Summary of physiological changes during healing with novel fibrin sealant.

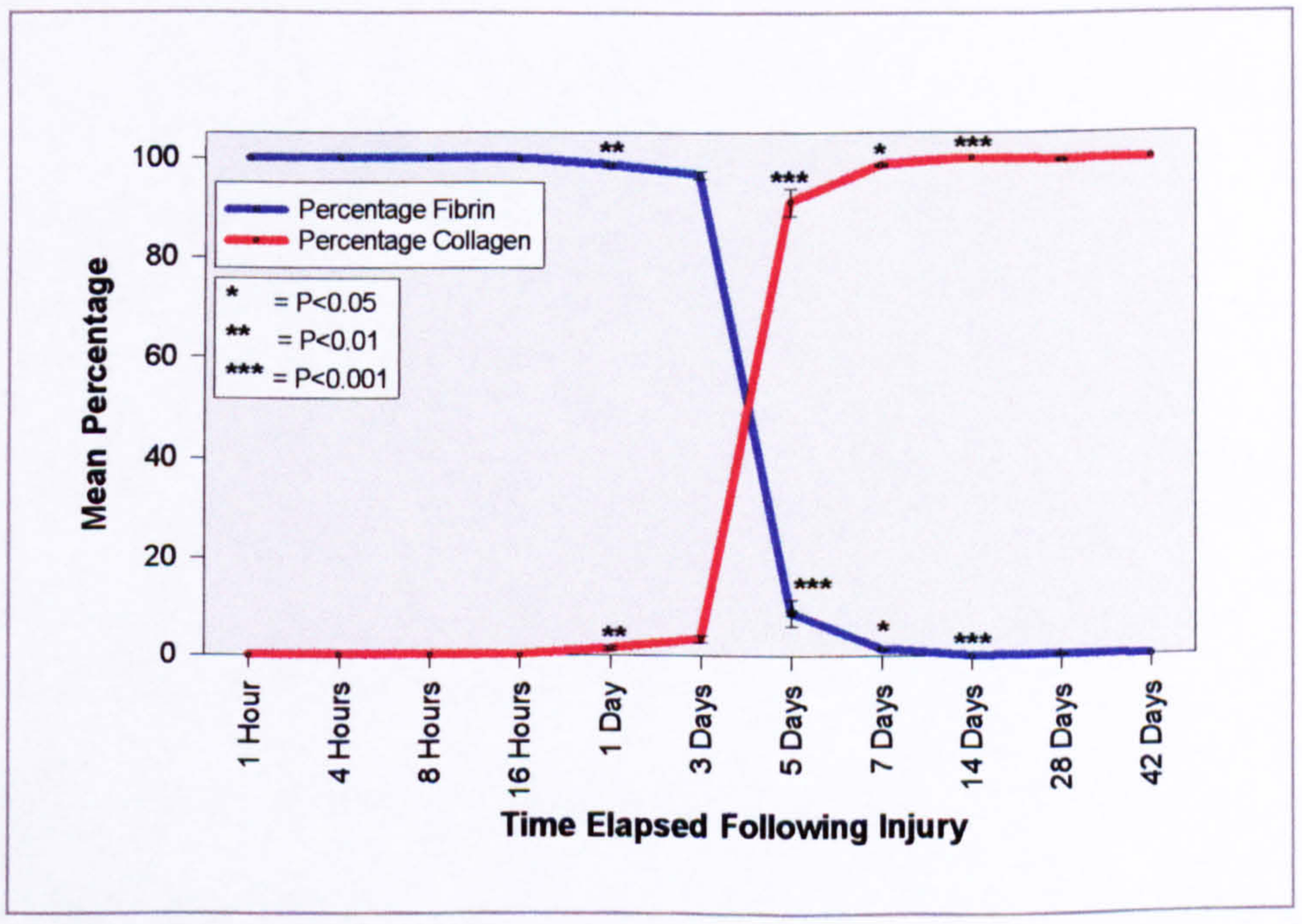
4.5 Volumetric Analysis of Fibrin and Collagen

In order to investigate mechanisms of action of PSA prevention by novel fibrin sealant, volumes of the main two structural proteins, fibrin and collagen, were studied. 5μm sections were cut from wax embedded blocks and stained with PTAH and/or Van Gieson for detection of fibrin and collagen respectively. Absolute volumes of fibrin and collagen in tissue generation and PSAs were determined using Cavalieri's Principle (refer to

Chapter 2).

4.5.1 Results

Fibrin was predominantly present in tissue generation from 1 hour to 3 days post injury (Graph 4.9; Appendix IV Table 4.3; Figure 4.8). From 16 hours to 14 days mean percentage of fibrin decreased, gradually between 16 hours and 3 days then rapidly between 5 and 14 days. Significant decreases occurred between 16 hours and 1 day ($P<0.01$), 3 and 5 days ($P<0.001$), 5 and 7 days ($P<0.05$) and 7 to 14 days ($P<0.001$) (Appendix IV Table 4.4). The mirror image was seen for mean percentage of collagen present: first seen at 1 day post injury, with a rapid increase from 3 to 5 days, and then predominant at 5 days and for the remainder of the study (Graph 4.9; Appendix IV Tables 4.3 and 4.4; Figure 4.9).



Graph 4.9: Mean fibrin and collagen percentages together with SEMs and statistical differences.

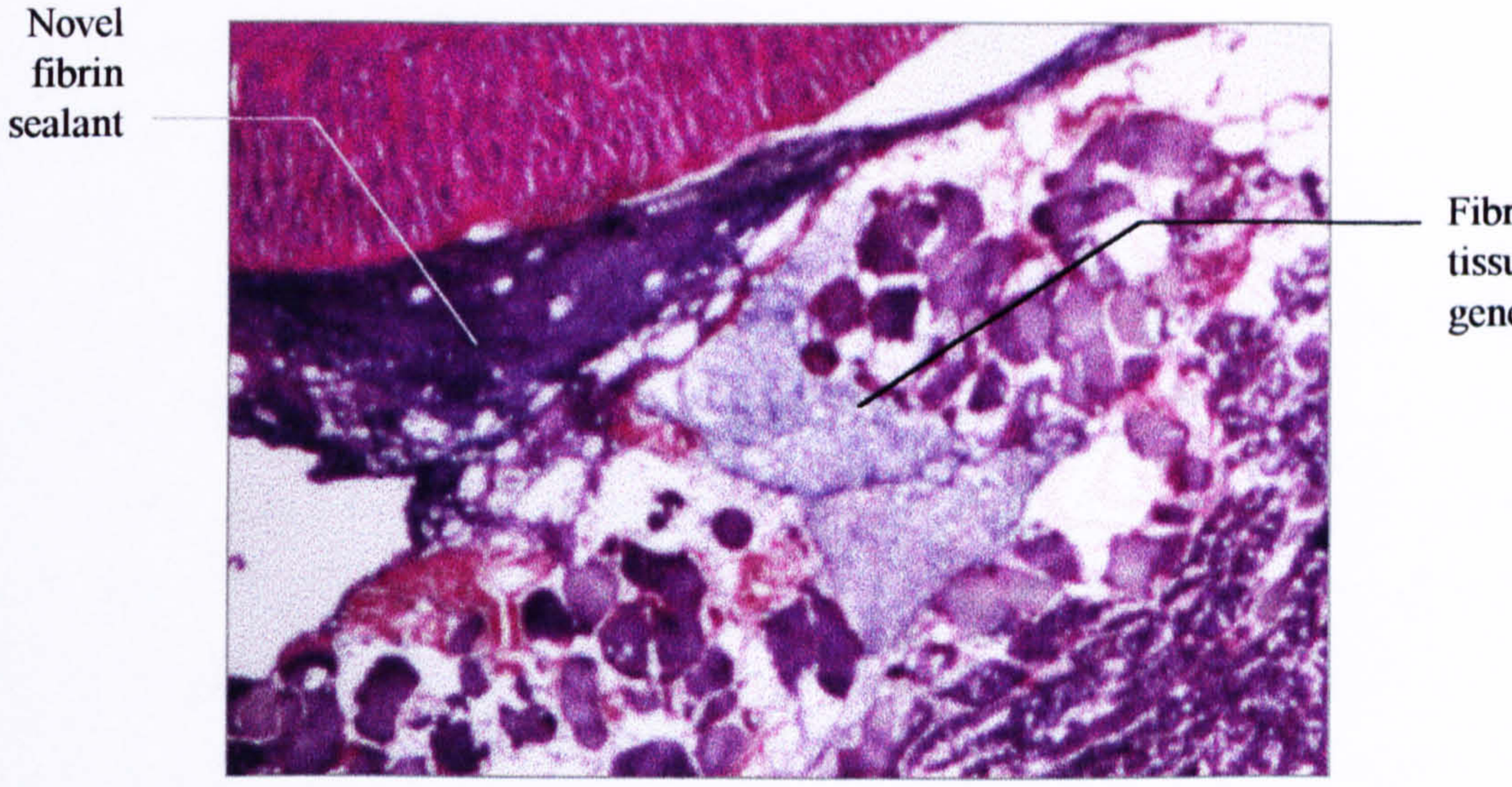


Figure 4.8:Fibrinous tissue generation
(1 day post injury) (M_o x4) (stained with PTAH)

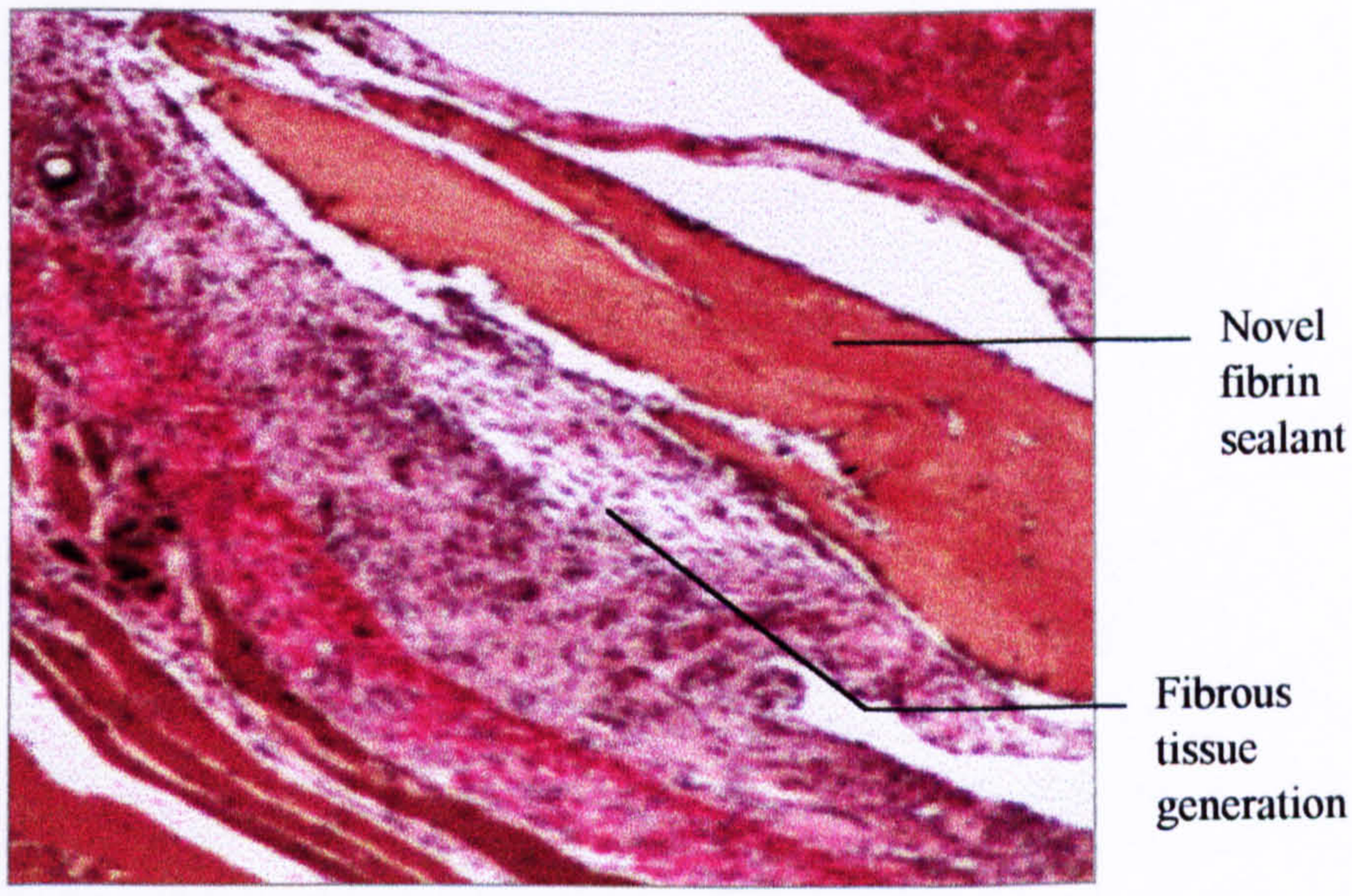


Figure 4.9:Fibrous tissue generation
(7 days post injury) (M_o x4) (stained with Van Gieson)

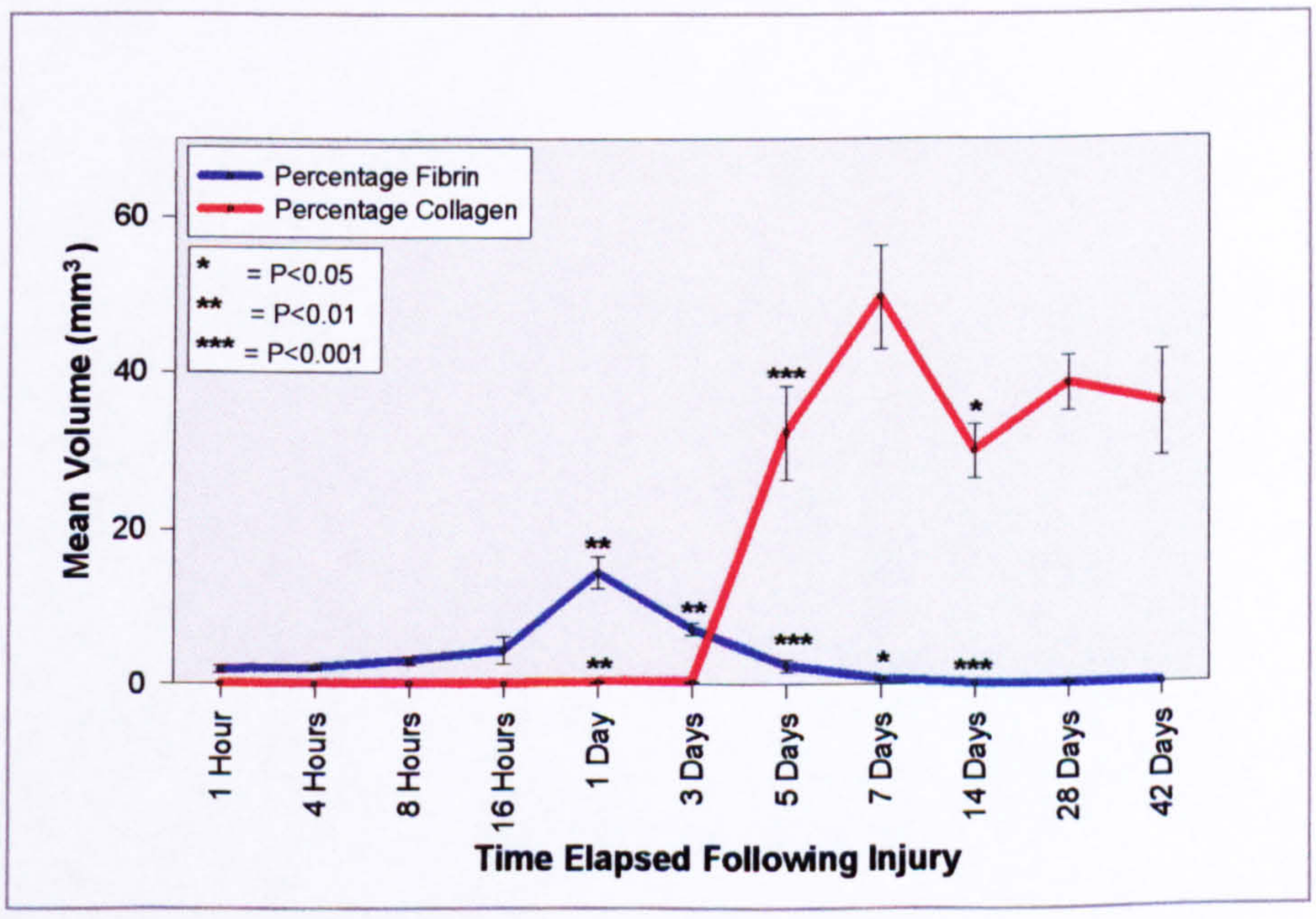
Mean volume of fibrin increased from 1 hour post injury to peak at 1 day, with a significant increase ($P<0.01$) between 16 hours and 1 day. From 1 day onwards mean volume decreased significantly ($P<0.05$) to become absent by 14 days (Graph 4.10; Appendix IV Tables 4.3 and 4.4).

Collagen mean volumes increased slightly from 16 hours to 1 day and then very rapidly to peak at 7 days (Graph 4.10; Appendix IV Table 4.3). Significant increases were seen at 1 ($P<0.01$) and 5 days ($P<0.001$) (Appendix IV Table 4.4). From 7 to 14 days

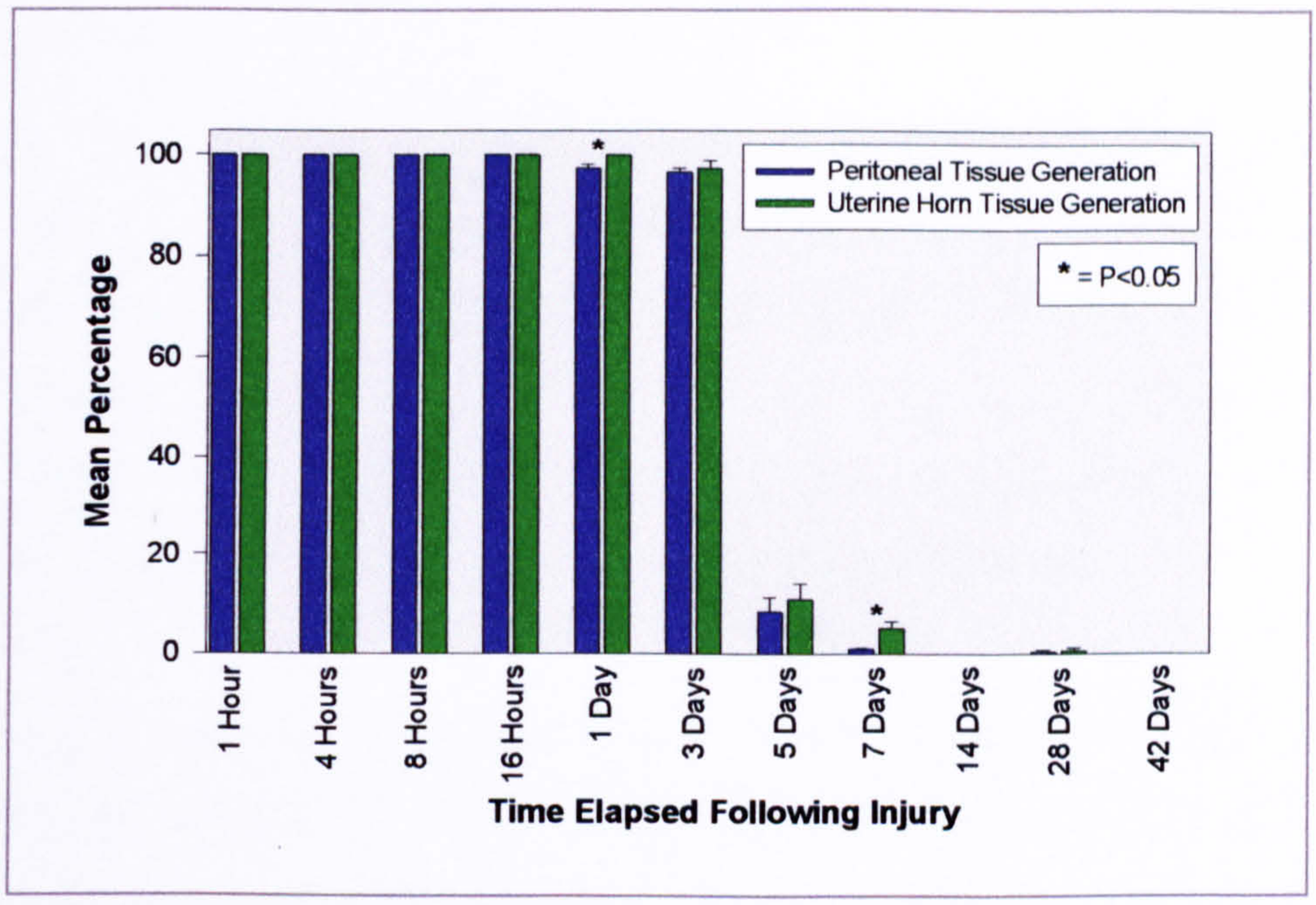
collagen volume decreased significantly ($P<0.01$) and then remained constant for the rest of the study.

Comparison of mean percentage of fibrin present within peritoneal and uterine horn tissue generation revealed similar trends throughout this study. Significant differences ($P<0.05$) were seen at 1 and 7 days post injury, with PTG having significantly lower mean volume of fibrin compared to UTG (Graph 4.11; Appendix IV Tables 4.3 and 4.4). The reverse situation was seen with mean percentage of collagen present in tissue generation: significantly greater percentage of collagen present in PTG compared to UTG at 1 and 7 days post injury (Graph 4.12; Appendix IV Tables 4.3 and 4.4).

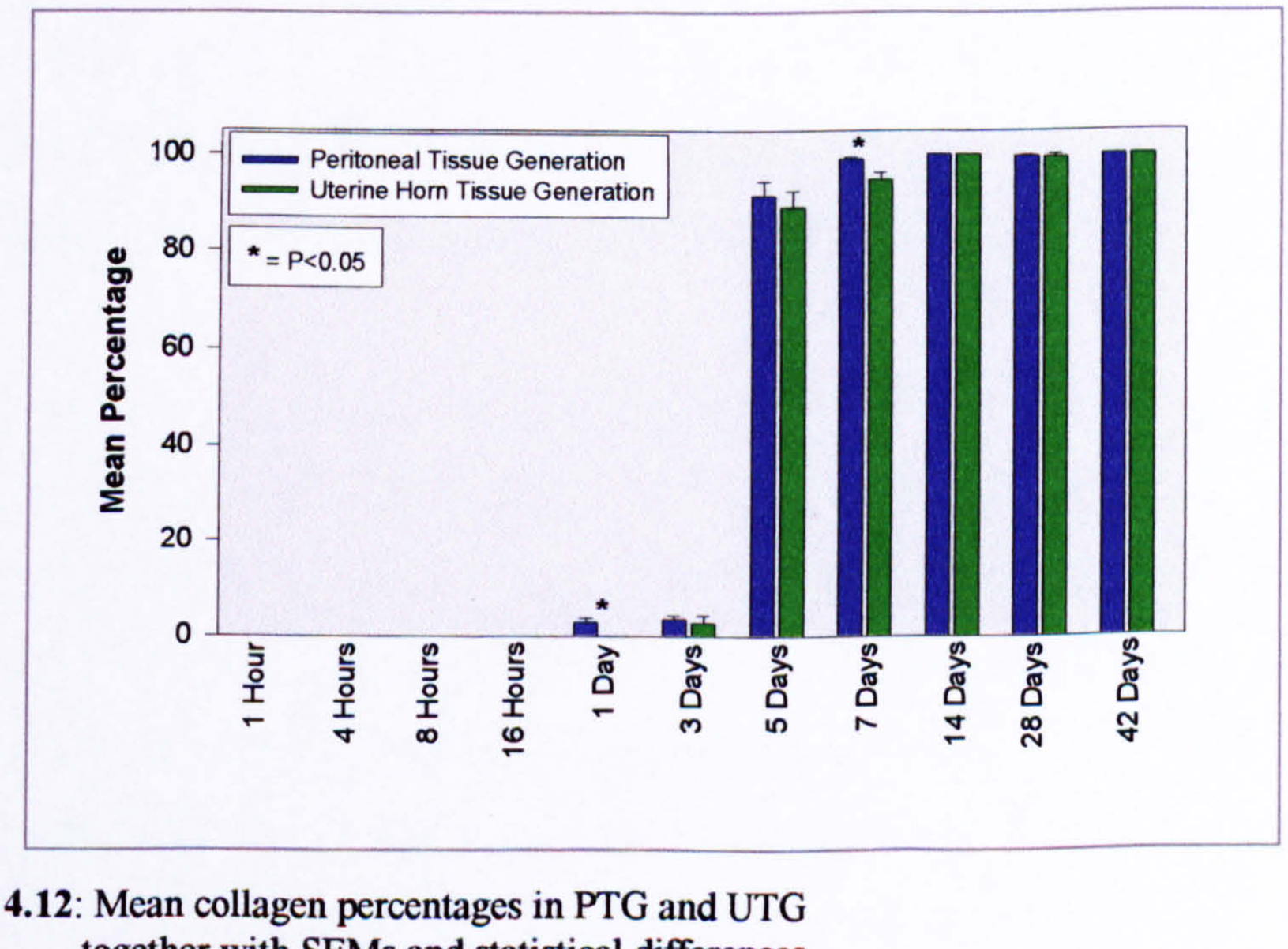
The mean coefficient of error for this study was 6.2%.



Graph 4.10: Mean fibrin and collagen volumes together with SEMs and statistical differences.



Graph 4.11: Mean fibrin percentages in PTG and UTG together with SEMs and statistical differences.



Graph 4.12: Mean collagen percentages in PTG and UTG together with SEMs and statistical differences.

4.5.2 Discussion

For the first 16 hours of tissue repair using novel fibrin sealant, fibrin only was present in tissue generated in response to injury. Fibrin remained present up to 7 days and was the predominant structural protein present up to/including 3 days post injury. Whereas collagen was first detected at 1 day and was the predominant protein from 5 days onwards. Hence tissue generated in response to injury in the presence of novel fibrin sealant was fibrinous up to 3 days post injury and fibrous thereafter.

This pattern and timing for the protein content of injury tissue corresponds with that seen with adhesion-free healing and PSA formation. In adhesion-free healing fibrin strands are seen rapidly at the site of injury, with complete coverage of the wound surface during the first 2 to 3 days, after which fibrin is eliminated from the wound site and collagen fibres are observed at 5 days onwards (Johnson & Whitting, 1962; Eskeland, 1966.1; Eskeland, 1966.2; Raftery, 1973.L; Raftery, 1973.E; Ryan *et al.*, 1973) (refer to section 1.1.6.3.2 of this thesis). An analogous situation was found for PSA composition by Milligan & Raftery (1974) (refer to section 1.1.6.5.1 of this thesis). Treatment with novel fibrin sealant at injury sites to prevent PSAs had no effect on the timing of fibrin and collagen predominance. That is, the general pattern of wound healing was unaffected.

The peak in volume of fibrinous tissue generation was seen at 1 day post injury, during the first day of wound healing with continuous fibrin deposition seen. There must therefore be a stimulatory effect from the site of injury for the further laying down of fibrin even when haemostasis has long been achieved. The origin of this stimulator is hypothesised to be cytokines and/or growth factors secreted from cells present at the site of injury. After 1 day there was a rapid and consistent decrease in the volume of fibrin present due to fibrinolysis and/or phagocytosis by inflammatory cells present at the wound site.

From 3 to 7 days following injury there was a very rapid lay down of collagen with peak volume of fibrous tissue generation at 7 days post injury. Deposition of collagen from fibroblasts was very rapid causing the conversion from fibrinous to fibrous injury tissue

in only a couple of days. Volume of fibrous tissue generation decreased from 7 to 14 days with little change thereafter. It is highly unlikely that collagen would have been lost from injury tissue and therefore the decrease in mean volume is due to contraction of collagen bundles within the tissue, which is a normal feature of wound healing (Ellis *et al.*, 1965). By 14 days post injury structural development and maturation of tissue generated in response to injury was completed.

Comparison of fibrin and collagen in PTG and UTG revealed significant differences at 1 and 7 days post injury, with PTG possessing lower percentages of fibrin and higher percentages of collagen compared to UTG at both time points. Although both types of tissue generation follow the same pattern as described above, collagen was seen in PTG at 1 day whereas it is not seen until 3 days within UTG. Since collagen is synthesised by fibroblasts it would appear that fibroblasts are present in peritoneal injury tissue before uterine horn injury tissue. The peritoneum has a connective tissue component within its structure which contains some fibroblasts which it appears are recruited to the injury tissue within the peritoneal wall. The uterine horn does not have this "connective tissue reservoir" and hence the arrival of fibroblasts through chemotaxis is later compared to the peritoneal wall. It is probably this "delay" in fibroblast presence and hence collagen synthesis which accounts for the greater presence of fibrin/reduced presence of collagen in UTG at 7 days post injury. By 14 days post injury both PTG and UTG tissues are composed completely of collagen and differences between them are eradicated by this time.

There were no significant differences seen between PSA, PTG and UTG in this study. However since there was only one case which demonstrated PSAs it is dubious as to whether any difference could be detected.

Conclusions

i. Tissue generated in response to injury in the presence of novel fibrin sealant was fibrinous up to/including 3 days and fibrous thereafter.
ii. Peak in fibrinous tissue generation was seen at 1 day and peak in fibrous tissue

generation at 7 days post injury.

iii. Development and maturation of this tissue was complete by 14 days post injury and followed the pattern for adhesion-free healing and PSA development.

iv. Decreased fibrin and increased collagen percentages in PTG compared to UTG at 1 and 7 days were due to fibroblast migration from the connective tissue in the peritoneal wall to the injury site.

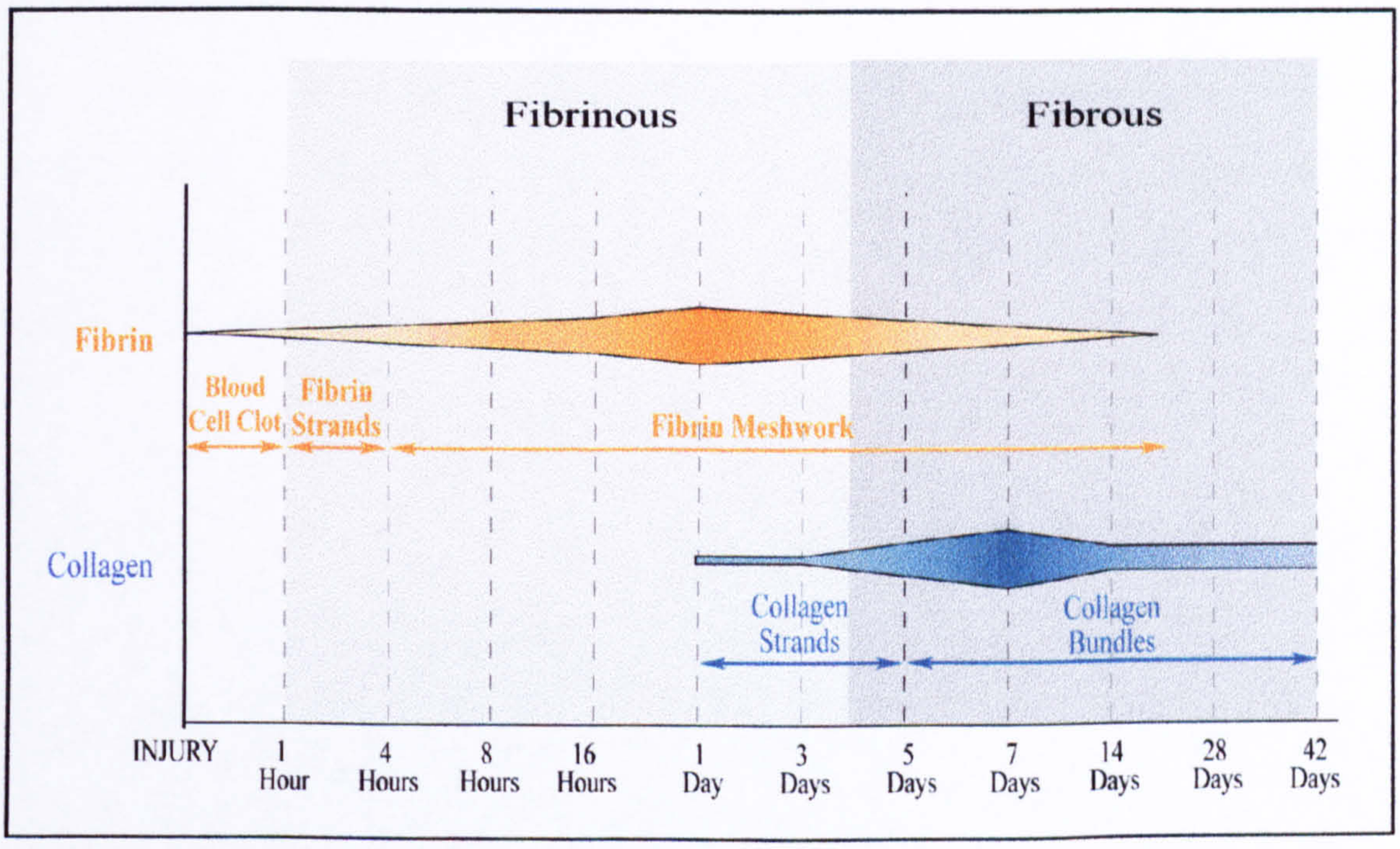


Figure 4.10: Summary of structural changes in injury tissue with the use of novel fibrin sealant.

4.6 Number Analysis of Cellular Components

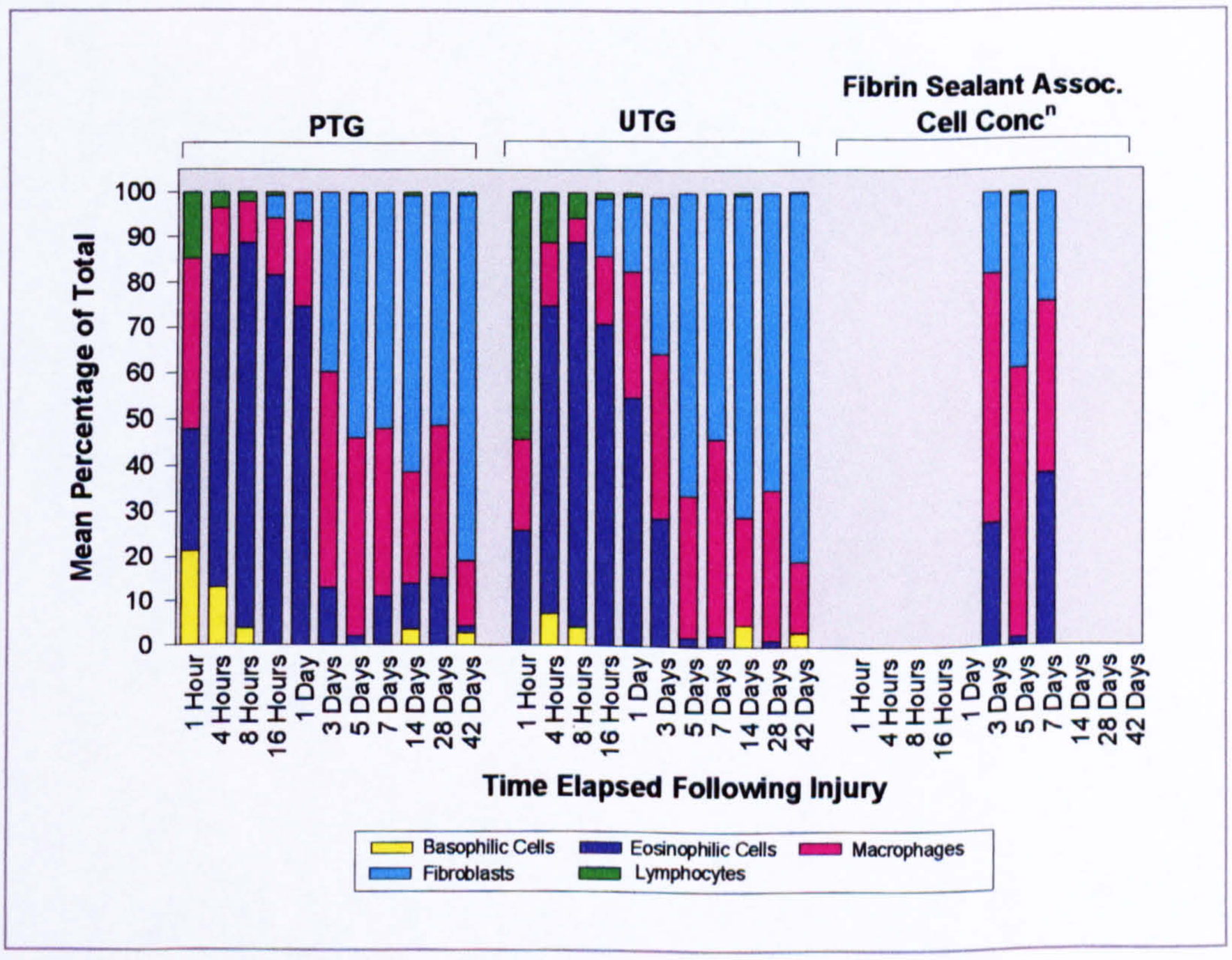
In order to gain an understanding of the mechanism(s) utilised by novel fibrin sealant to prevent PSAs, cellular presence and activity at the site of injury is a necessity. Number densities and totals of basophilic cells, eosinophilic cells, macrophages, fibroblasts and lymphocytes within PTG, UTG, PSA and fibrin sealant associated cell concentration were determined using a stereological technique, the optical brick. Even numbered slices of fixed resected tissues were processed to resin, with a 25μm section cut from each block and stained with a modified Giemsa stain (refer to Chapter 2).

4.6.1 Results

4.6.1.1 Number Densities

4.6.1.1.1 Cell Predominance

Both PTG and UTG demonstrated a similar pattern with regard to cellular presence throughout this study. Up to and including 1 day post injury eosinophilic cells were predominant within injury tissue (Figure 4.11) and from 3 to 5 days onwards fibroblasts were the predominant cell type present (Graph 4.13; Appendix IV Table 4.7; Figure 4.12). Whereas macrophages were predominant in fibrin sealant associated cell concentration at time points analysed (3 to 7 days), followed by either eosinophilic cells and fibroblasts (3 and 7 days) or fibroblasts and eosinophilic cells (5 days) (Graph 4.13; Appendix IV Table 4.7).



Graph 4.13: Number density cell predominances in tissue generation and fibrin sealant associated cell concentration.

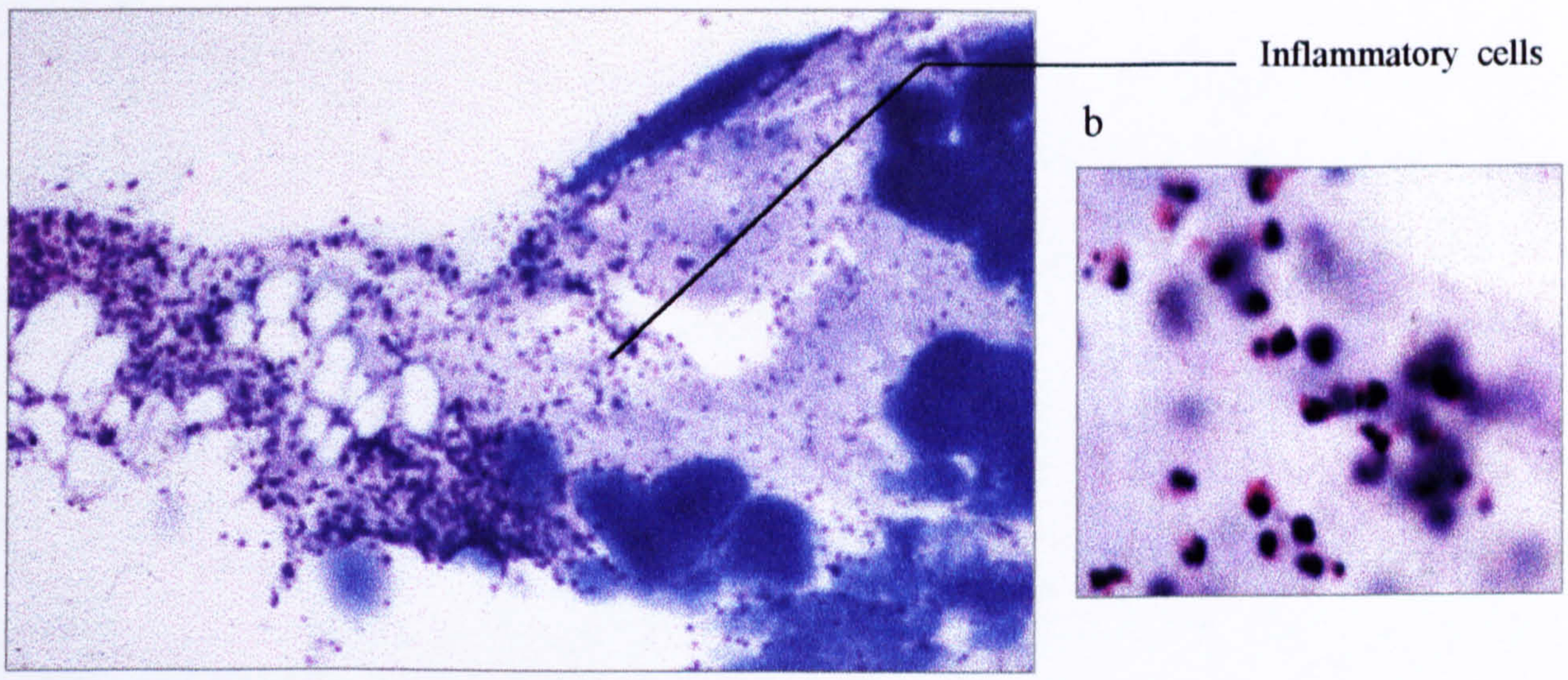


Figure 4.11 : Inflammatory cell predominance at 16 hours post injury
(a = Mo x 10, b= Mo x 100)

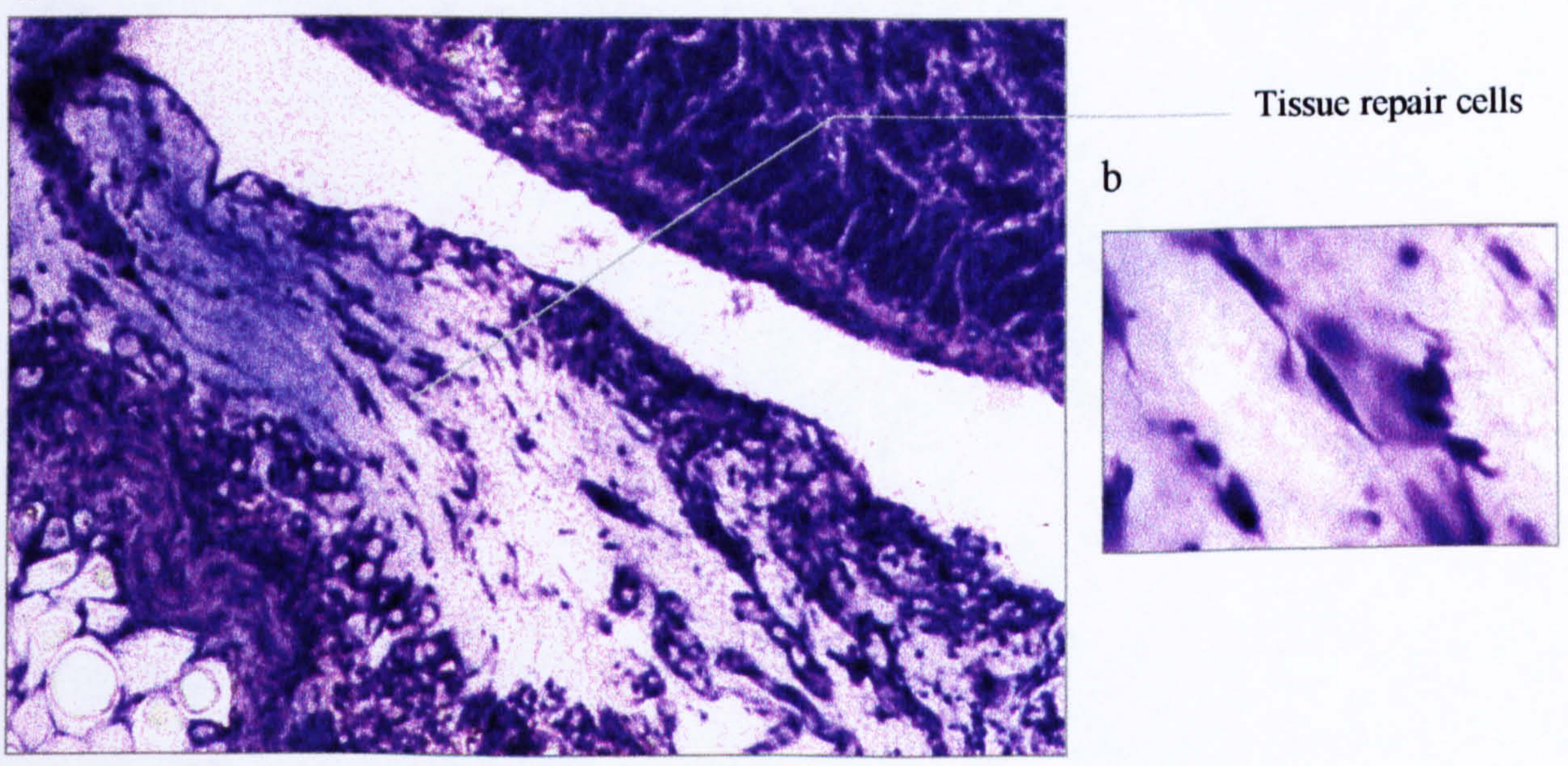


Figure 4.12 : Tissue repair cell predominance at 5 day post injury
(a = Mo x 10, b= Mo x 100)

4.6.1.1.2 Post Surgical Adhesion

Of the two experimental sites which demonstrated PSAs only one site had PSA available for number density analysis following resin processing. This 28 day case had a total number density of 454 167 cells per cubic millimetre of PSA, with fibroblasts accounting for 68%, macrophages 29% and eosinophilic cells 3%. No basophilic cells or lymphocytes were present within PSA of this case (Appendix IV Table 4.6).

4.6.1.1.3 Peritoneal Tissue Generation

Number density of total cells present within PTG increased significantly ($P<0.05$) and uniformly from 1 to 16 hours post injury (Graph 4.14; Appendix IV Tables 4.7 and 4.8). From 16 hours to 3 days little change was seen but subsequent peaks in number density were seen at 5 and 14 days. From 14 days until the completion of this study number density decreased with a significant decrease ($P<0.05$) between 28 and 42 days.

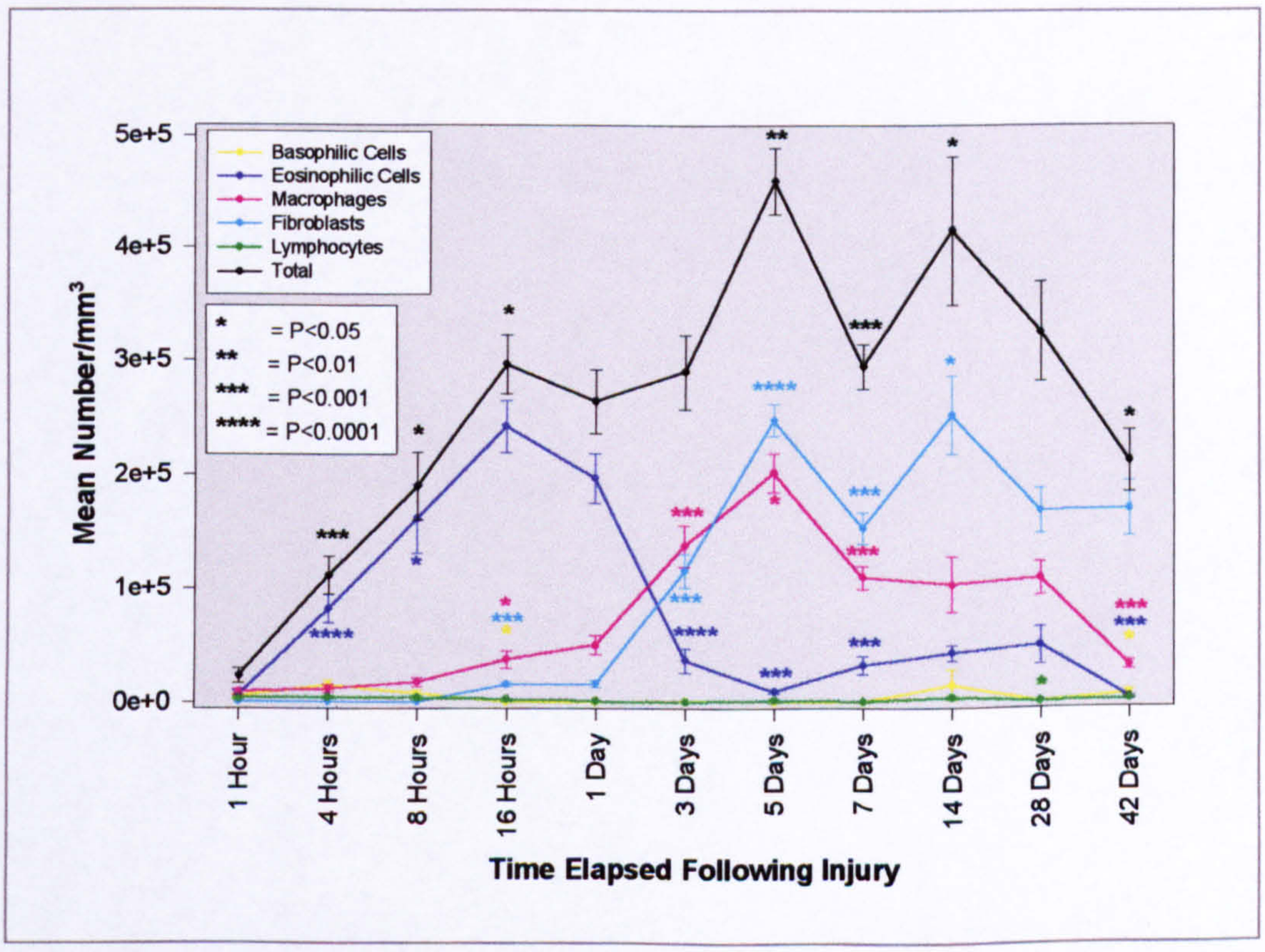
Number density of basophilic cells demonstrated peaks at 4 hours and 14 days post injury (Graph 4.14; Appendix IV Tables 4.7 and 4.8). Little variation was seen at the intermediate time points, however, significant decreases ($P<0.05$) were present between 8 and 16 hours and 28 and 42 days. Eosinophilic cells demonstrated a rapid increase in mean number density from 1 to 16 hours with significant increase between 1 and 4 hours ($P<0.0001$) and 4 and 8 hours ($P<0.05$) post injury (Graph 4.14; Appendix IV Tables 4.7 and 4.8). Following the peak density at 16 hours, eosinophilic cell number density decreased steadily to 5 days and significant decreases between 1 and 3 days ($P<0.0001$) and 3 and 5 days ($P<0.001$). Number density then showed a trend to increase slightly from 5 to 28 days, with a significant increase ($P<0.001$) between 5 and 7 days and levels decreasing significantly ($P<0.001$) at the end of the study.

Macrophage number density increased steadily from 1 hour to 1 day with a significant increase ($P<0.05$) between 8 and 16 hours (Graph 4.14; Appendix IV Tables 4.7 and 4.8). This trend to increase was accelerated between 1 and 5 days with significant increases ($P<0.05$) throughout this time period. Following the number density peak at 5 days a significant decrease ($P<0.001$) occurred between 5 and 7 days, with constant density between 7 and 28 days, and finally a significant decrease ($P<0.001$) from 28 to 42 days.

Fibroblasts were first evident within PTG at 16 hours post injury, with a significant difference ($P<0.001$) between 8 and 16 hours (Graph 4.14; Appendix IV Tables 4.7 and 4.8). A slight increase in fibroblast number density was seen from 16 hours to 1 day after which there was a rapid and significant increase ($P<0.001$) until 5 days. A transient drop

occurred at 7 days ($P<0.001$) with a second peak at 14 days ($P<0.05$). Fibroblast number density then decreased from 14 to 28 days, with constant levels seen until the end of this study.

Very little variation was seen for lymphocyte number density within PTG throughout this study (Graph 4.14; Appendix IV Tables 4.7 and 4.8). Mean density demonstrated a trend to be greater during the first 16 hours and a significant decrease ($P<0.05$) was evident between 14 and 28 days post injury.



Graph 4.14: Mean cell densities throughout PTG development together with SEMs and statistical differences.

4.6.1.1.4 Uterine Horn Tissue Generation

Number density of total cells within UTG gradually increased from 1 hour to 1 day and then rapidly from 1 to 5 days ($P<0.01$) with density peaking at this time (Graph 4.15; Appendix IV Tables 4.7 and 4.8). A significant decrease ($P<0.001$) was seen between 5 and 7 days and density remained unchanged from 7 days until the end of this study.

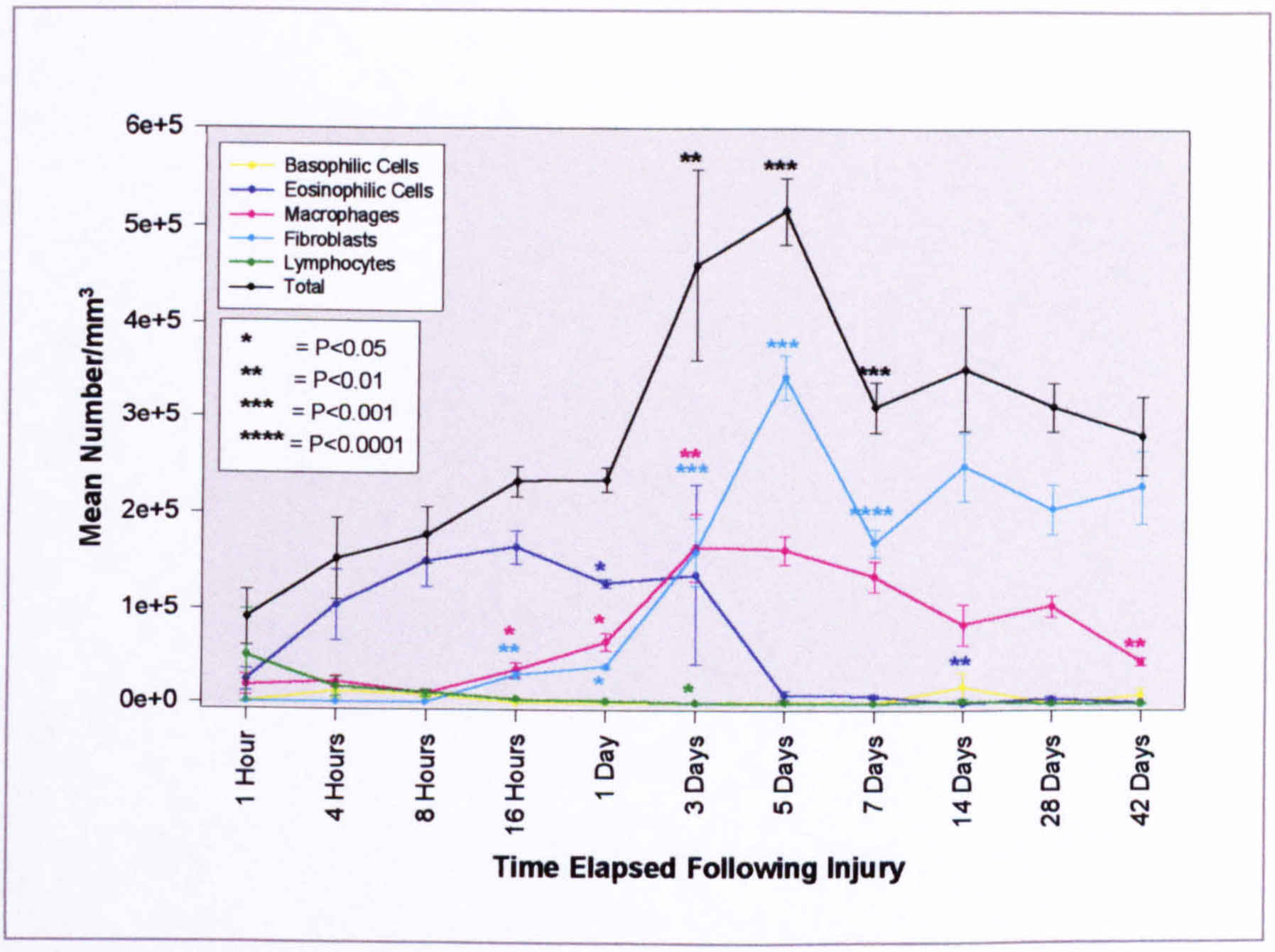
Number density of basophilic cells within UTG remained relatively constant through the

whole of this study with no significant differences (P>0.05) (Graph 4.15; Appendix IV Tables 4.7 and 4.8). However transient peaks at 4 hours and 14 days were seen. In contrast eosinophilic cells demonstrated noticeable trends in number density throughout the course of this study. From 1 to 16 hours mean density increased, with elevated levels sustained until 3 days following injury, however, a significant decrease (P<0.05) was seen between 16 hours and 1 day (Graph 4.15; Appendix IV Tables 4.7 and 4.8). From 3 to 5 days there was a rapid decrease with density remaining minimal for the remainder of the study and a significant decrease (P<0.01) between 7 and 14 days.

There was little change in macrophage number density within UTG for the first 8 hours following injury, however from 8 hours to 3 days mean density increased significantly (P<0.05) to a peak (Graph 4.15; Appendix IV Tables 4.7 and 4.8). From 3 until 42 days mean macrophage density gradually decreased, with a significant decrease (P<0.01) at the end of this study.

16 hours post injury was the first time fibroblasts were detected within UTG with density significantly increasing (P<0.05) from this time until 5 days (Graph 4.15; Appendix IV Tables 4.7 and 4.8). Mean fibroblast density then significantly decreased (P<0.0001) from 5 to 7 days, with density slightly increasing from 7 to 14 days and remaining unchanged from thereon.

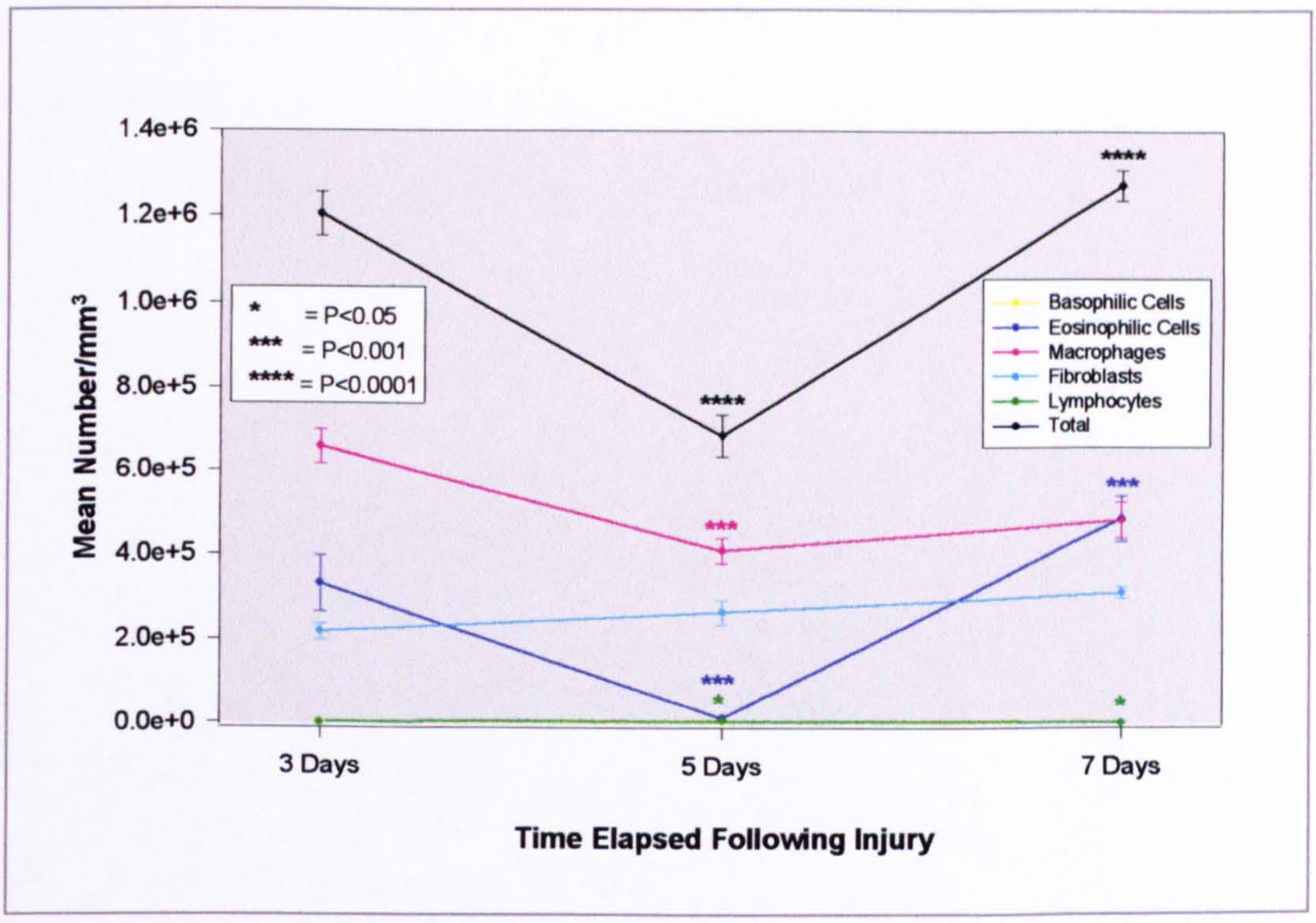
Lymphocyte density within UTG were at their greatest at 1 hour following injury with density from 1 hour to 1 days demonstrating a trend to be greater compared to the rest of the study (Graph 4.15; Appendix IV Tables 4.7 and 4.8). Only one significant difference (P<0.05) was seen throughout the course of this study, a significant decrease (P<0.05) from 1 to 3 days.



Graph 4.15: Mean cell densities throughout UTG development together with SEMs and statistical differences.

4.6.1.1.5 Fibrin Sealant Associated Cell Concentration

Number density of total cells analysed within fibrin sealant associated cell concentration demonstrated similar values at 3 and 7 days post injury, with a significant trough at 5 days ($P<0.0001$) (Graph 4.16; Appendix IV Tables 4.7 and 4.8). Number density of basophilic cells demonstrated a trend to decline from 3 to 7 days. Eosinophilic cells followed the pattern seen for total cells, alike values at 3 and 7 days with a significant decrease ($P<0.001$) at 5 days following injury. Mean macrophage density showed a significant decrease ($P<0.001$) from 3 to 5 days, with this level maintained at 7 days. Fibroblasts showed little variation throughout this time period, with a very slight trend to increase whereas lymphocytes were absent at 3 and 7 days but present in low densities at 5 days following injury (Graph 4.16; Appendix IV Tables 4.7 and 4.8).



Graph 4.16: Mean cell densities within fibrin sealant associated cell concentration together with SEMs and statistical differences.

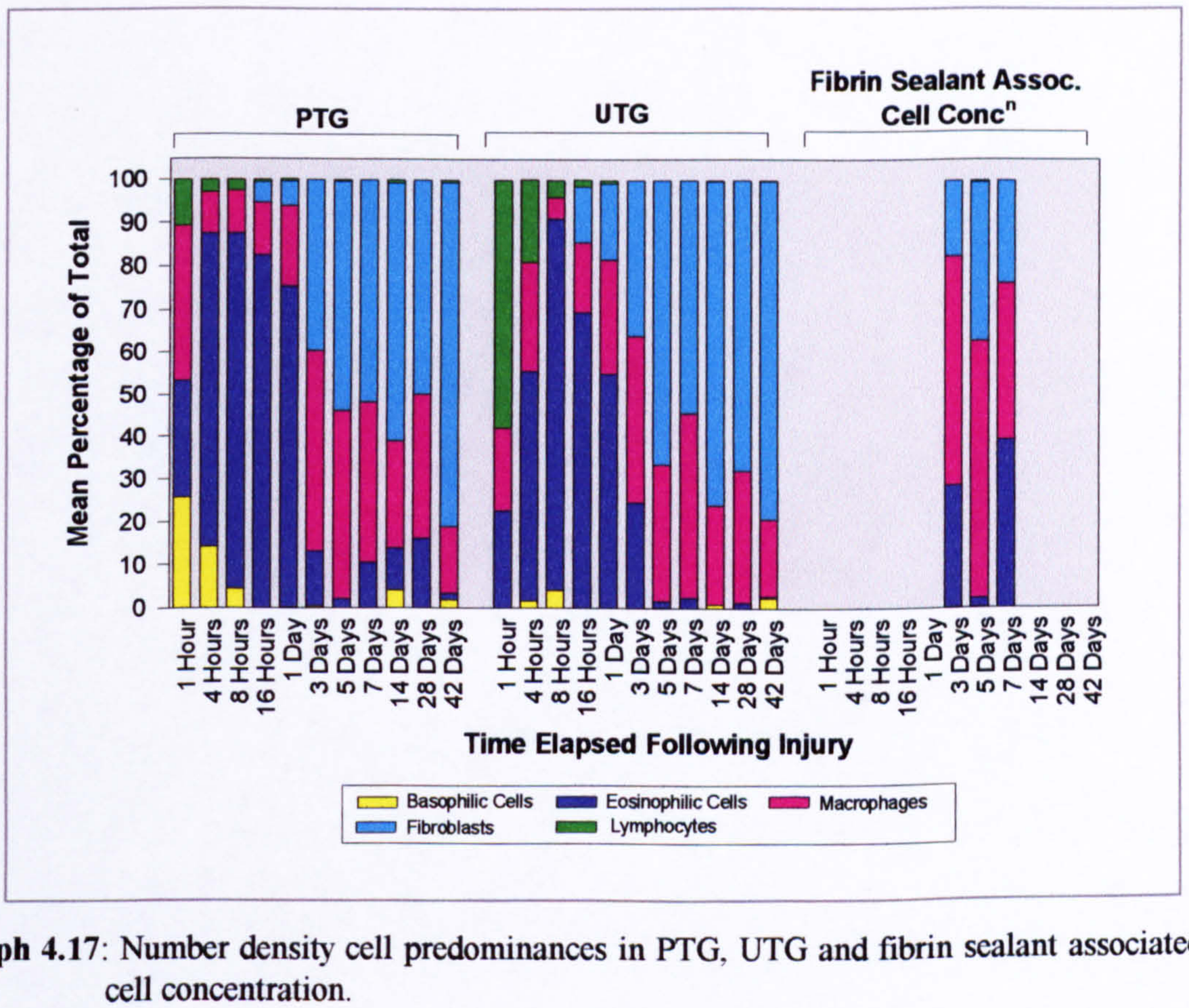
Comparison of number densities within PTG and UTG revealed differences for total cells, eosinophilic cells, macrophages and fibroblasts (Appendix IV Table 4.9). At the first sample time point of 1 hour PTG contained significantly greater ($P<0.05$) densities of cells (totals) compared to UTG. At 16 hours, 1, 7, 14 and 28 days post injury eosinophilic cell number densities were significantly greater ($P<0.05$) within PTG compared to UTG. Whereas at 42 days macrophage number densities were significantly greater ($P<0.05$) within UTG compared to PTG and 16 hours, 1 and 5 days UTG had significantly greater (P0.05) densities of fibroblasts compared to PTG.

4.6.1.2 Number Totals

4.6.1.2.1 Cell Predominance

The same pattern of cell predominance in tissue generation was seen with number totals as number densities. Eosinophilic cells were present in greatest number up to 1 day following injury after which fibroblasts were the predominant cell type (Graph 4.17; Appendix IV Table 4.10).

Within fibrin sealant associated cell concentration macrophages were the predominant cell type, followed by eosinophilic cells and then fibroblasts at 3 and 7 days and vice versa at 5 days post injury (Graph 4.17; Appendix IV Table 4.10).



Graph 4.17: Number density cell predominances in PTG, UTG and fibrin sealant associated cell concentration.

4.6.1.2.2 Post Surgical Adhesion

There were 15 350 745 total cells analysed within the PSA present in this study (Appendix IV Table 4.6). Percentage breakdown of each cell type is the same as that for number density: 68% fibroblasts, 29% macrophages and 3% eosinophilic cells.

4.6.1.2.3 Peritoneal Tissue Generation

Total cell numbers within PTG demonstrated a steady increase from 1 hour to 3 days, with significant increases between 1 and 4 hours ($P<0.001$), 4 and 8 hours ($P<0.01$), and 16 hours and 1 day ($P<0.01$). From 3 to 5 days there was a massive significant increase ($P<0.01$) in total cells, peaking at this later time point and decreasing thereafter and a significant decrease ($P<0.05$) from 28 to 42 days post injury (Graph 4.18; Appendix IV

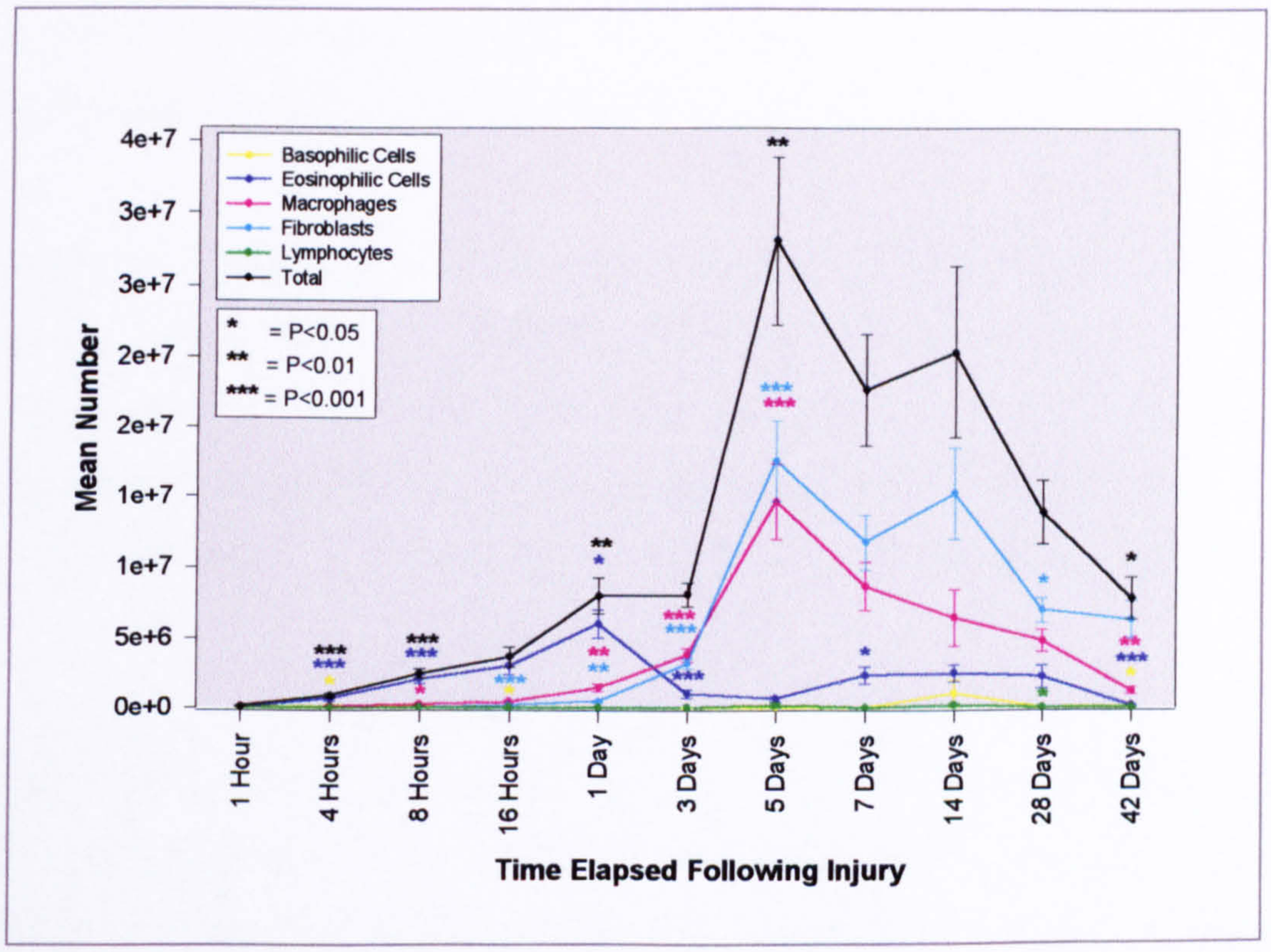
Tables 4.10 and 4.11).

Numbers of basophilic cells within PTG were elevated at 4 and 8 hours and 14 days post injury (Graph 4.18; Appendix IV Tables 4.10 and 4.11). Significant increases ($P<0.05$) were present between 1 and 4 hours and 28 and 42 days and a significant decrease ($P<0.05$) between 8 and 16 hours. Eosinophilic cell numbers increased constantly from 1 hour to 1 day, being significant between 1 and 4 hours ($P<0.001$), 4 and 8 hours ($P<0.001$) and 16 hours and 1 day ($P<0.05$) (Graph 4.18; Appendix IV Tables 4.10 and 4.11). Following the peak at 1 day, mean numbers of eosinophilic cells decreased significantly ($P<0.001$) to 3 days, remained unchanged at 5 days and then demonstrated a significant increase ($P<0.05$) at 7 days. From 7 to 28 days there was little change in mean number, however, a significant decrease ($P<0.001$) was seen between 28 and 42 days post injury.

Macrophage mean numbers within PTG increased from the first time point of 1 hour to peak at 5 days following injury, with this increase demonstrating an exponential nature and significant increases between 4 and 8 hours ($P<0.05$), 16 hours and 1 day ($P<0.01$), 1 and 3 days ($P<0.001$) and 3 and 5 days ($P<0.001$) (Graph 4.18; Appendix IV Tables 4.10 and 4.11). From 5 days onwards mean numbers decreased, being significant ($P<0.01$) between 28 and 42 days.

Mean numbers of fibroblasts present within PTG followed a similar pattern to macrophages. They were first present at 16 hours post injury and increased significantly ($P<0.01$) from this time to peak at 5 days (Graph 4.18; Appendix IV Tables 4.10 and 4.11). From 5 to 42 days mean numbers decreased, with a slight increase at 7 days and a significant decrease ($P<0.05$) between 7 and 14 days.

Lymphocytes were present throughout this study, with only minor fluctuations in mean number and no significant differences ($P>0.05$) (Graph 4.18; Appendix IV Tables 4.10 and 4.11).



Graph 4.18: Mean cell numbers throughout PTG development together with SEMs and statistical differences.

4.6.1.2.4 Uterine Horn Tissue Generation

Total numbers of cells analysed with UTG remained unchanged from 1 to 4 hours following injury but increased rapidly and significantly ($P<0.05$) from 4 hours to 1 days (Graph 4.19; Appendix IV Tables 4.10 and 4.11). From 1 to 3 days there was a significant decrease ($P<0.05$) in mean total number immediately followed by a significant increase ($P<0.05$) to peak for a second time at 5 days. From 5 days to the end of this study mean numbers decreased steadily, with a significant decrease ($P<0.05$) between 7 and 14 days and a slight peak at 28 days.

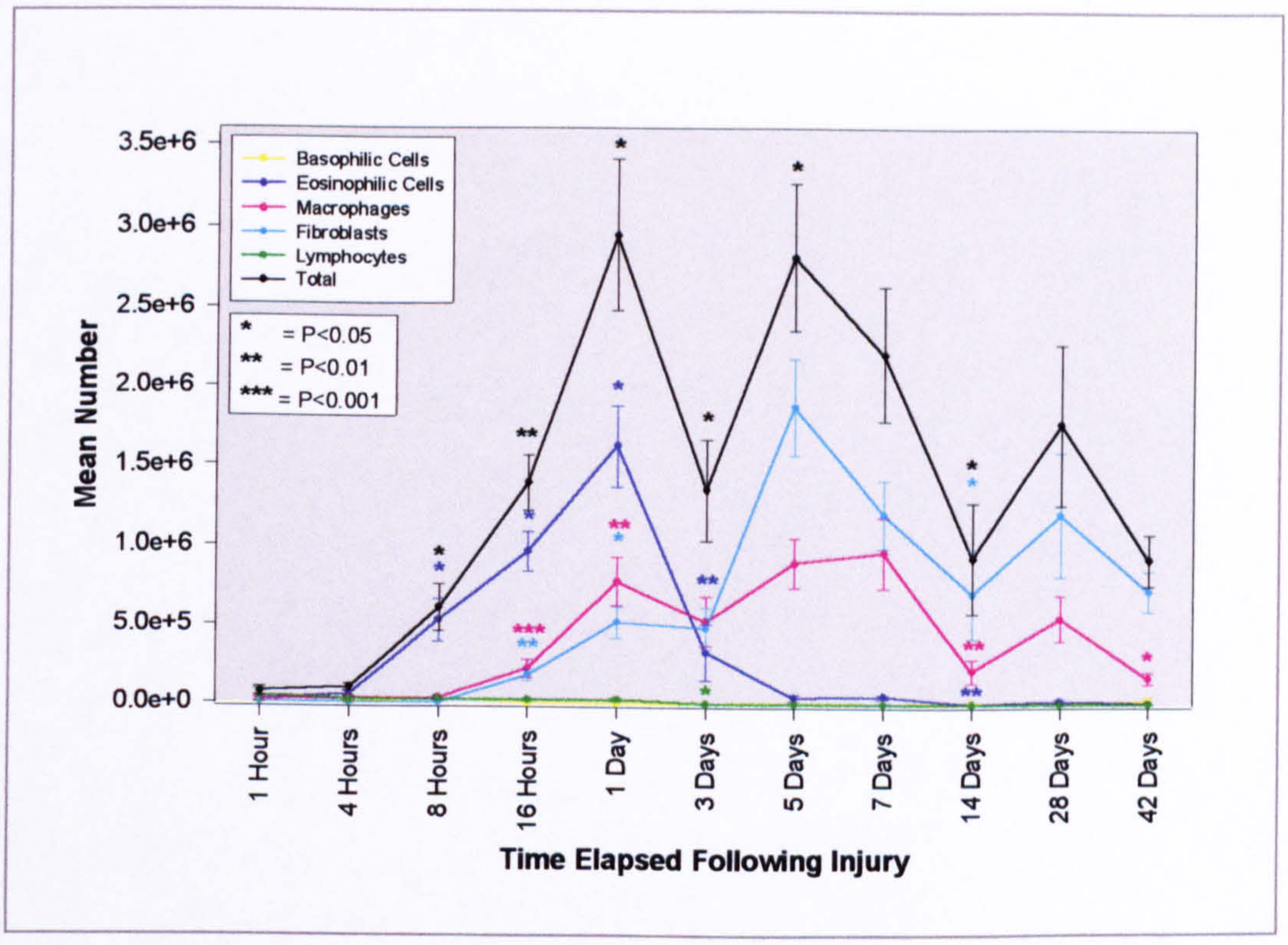
There were no significant differences ($P<0.05$) between any concurrent time point for mean numbers of basophilic cells present in UTG (Graph 4.19; Appendix IV Tables 4.10 and 4.11) although mean numbers demonstrated slight elevations at 8 hours and 42 days post injury. Little variation was seen for eosinophilic cell mean numbers between 1 and 4 hours however, this was followed by a rapid and significant increase ($P<0.05$) to peak at 1 day post injury (Graph 4.19; Appendix IV Tables 4.10 and 4.11). An equally rapid decrease occurred from 1 to 5 days, with a significant decrease ($P<0.01$) between 1 and

3 days. From 5 to 42 days an overall decrease in mean numbers of eosinophilic cells within UTG was seen with a significant decrease ($P<0.01$) between 7 and 14 days.

Mean numbers of macrophages within UTG showed a significant increase ($P<0.01$) from 8 hours to 1 day (Graph 4.19; Appendix IV Tables 4.10 and 4.11). Levels remained relatively constant between 1 and 7 days and then showed a trend to decrease until the end of this study. Significant decreases occurred between 7 and 14 days ($P<0.01$) and 28 and 42 days ($P<0.05$) and a slight but not significant ($P>0.05$) increase was seen at 28 days.

Fibroblast mean numbers increased gradually from 16 hours to 3 days and then rapidly to peak at 5 days. Significant increases occurred between 8 and 16 hours ($P<0.01$) and 16 hours and 1 day ($P<0.05$). From the peak at 5 days following injury mean numbers of fibroblasts decreased to 14 days, being significant ($P<0.05$) between 7 and 14 days and a slight increase at 28 days and then decrease to 42 days (Graph 4.19; Appendix IV Tables 4.10 and 4.11).

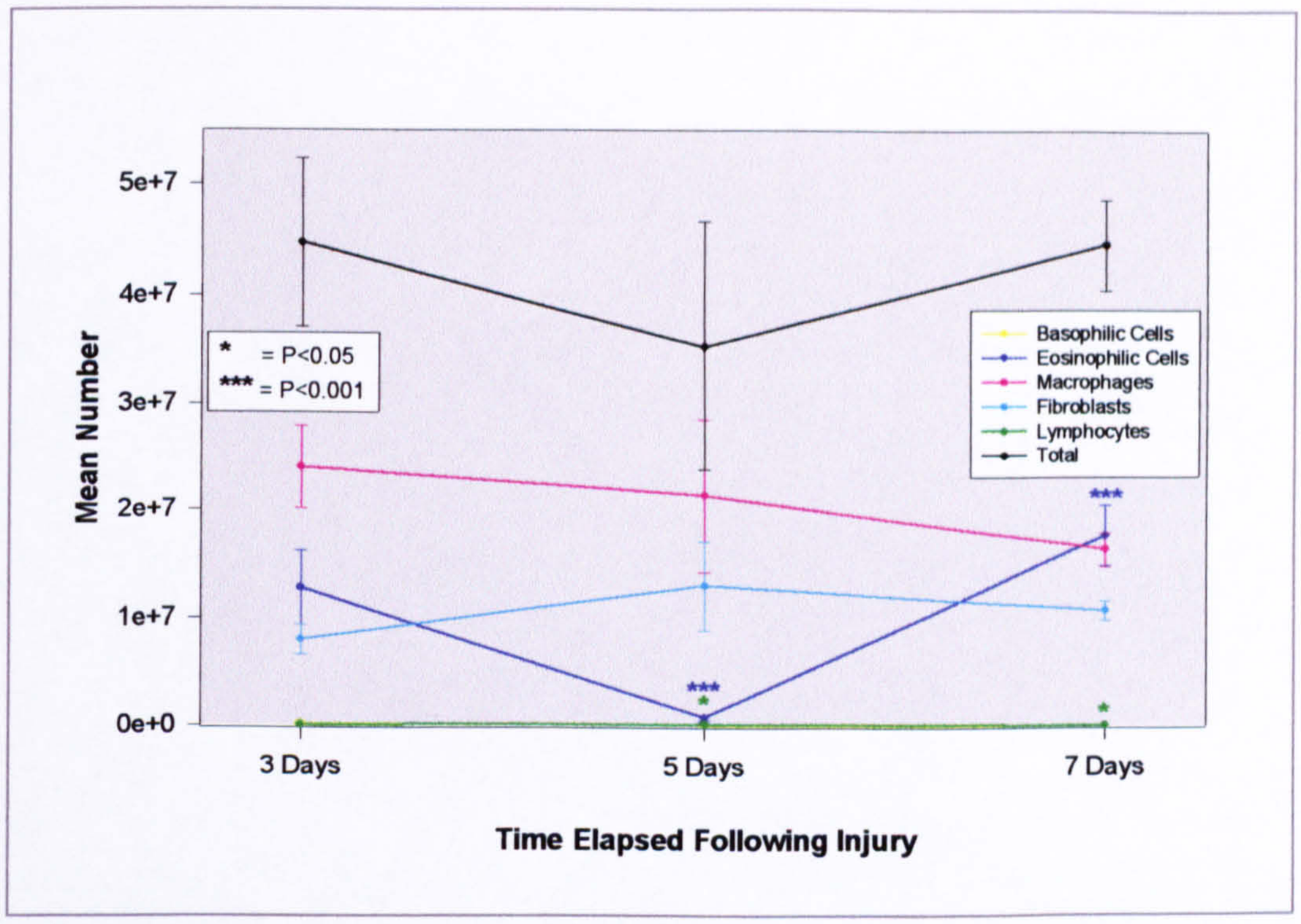
Mean numbers of lymphocytes were higher for the first day of this study compared with other time points, with a significant decrease ($P<0.05$) between 1 and 3 days post injury (Graph 4.19; Appendix IV Tables 4.10 and 4.11). Numbers remained similar for the rest of the study.



Graph 4.19: Mean cell numbers throughout UTG development together with SEMs and statistical differences.

4.6.1.2.5 Fibrin Sealant Associated Cell Concentration

Mean total numbers of cells within fibrin sealant associated cell concentration were not significantly different ($P>0.05$) from 3 to 7 days post injury (Graph 4.20; Appendix IV Tables 4.10 and 4.11). Basophilic cells demonstrated a reduction in mean numbers present between 3 to 7 days. Mean numbers of eosinophilic cells decreased significantly ($P<0.001$) from 3 to 5 days and then increased significantly ($P<0.001$) from 5 to 7 days. Macrophages demonstrated a trend to decrease in mean number from 3 to 7 days, whereas fibroblasts showed a trend to increase during the same time period. Lymphocytes were absent at 3 and 7 days post injury within fibrin sealant associated cell concentration but present at 5 days.



Graph 4.20: Mean cell numbers within fibrin sealant associated cell concentration together with SEMs and statistical differences.

Comparison of mean numbers of cells within PTG and UTG revealed significant differences ($P<0.05$) for all cell types except lymphocytes (Appendix IV Table 4.12). PTG contained significantly greater ($P<0.05$) numbers of cells compared to UTG for: basophilic cells at 4 hours post injury, eosinophilic cells from 4 hours to 28 days, macrophages from 4 to 8 hours and 1 to 42 days, fibroblasts from 3 to 42 days and total cells from 4 hours to 42 days.

The overall coefficient of error of this counting technique was 13.4%.

4.6.2 Discussion

The involvement of inflammatory cells in adhesion-free healing is well documented. They are present from 1 hour following injury, with macrophages being predominant together with some mast cells (Eskeland, 1966.1; Eskeland, 1966.2). By 12 hours, numbers of cells present have increased, with PMNLs predominant followed by macrophages, eosinophilic cells and mast cells respectively (Raftery, 1973. E). By 1 day a whole variety of inflammatory cells have been reported as present: neutrophils, eosinophils (Johnson &

Whitting, 1962; Hubbard *et al.*, 1967; Ryan *et al.*, 1973; Raftery, 1973 E), lymphocytes, histiocytes (Hubbard *et al.*, 1967) and macrophages (Johnson & Whitting, 1962; Hubbard *et al.*, 1967; Raftery, 1973 L; Raftery, 1973 E). At 3 days the general consensus is that macrophages are the only inflammatory cells present (Johnson & Whitting, 1962; Raftery, 1973 L; Raftery, 1973 E) with all inflammatory cells decreasing in number from 7 to 10 days (Raftery, 1973 E). With regard to fibroblasts, they have been reported at 2 days following injury (Ellis *et al.*, 1965), with proliferation at 4 days (Johnson & Whitting, 1962; Raftery, 1973 L) and their presence still evident at 10 days (Raftery, 1973 E).

The results of this study for adhesion-free healing with novel fibrin sealant are in agreement with these reported above. During the first day following injury inflammatory cells were the predominant cell types present in both peritoneal and uterine horn tissue generation. Eosinophilic cells were present in greatest percentages followed by basophilic cells and then macrophages. However between 1 and 3 days there was a change from inflammatory cell predominance to tissue repair cell predominance, with fibroblasts and then macrophages present in greatest percentages.

With regard to number densities within PTG the peak in inflammatory cells, due primarily to a peak in eosinophilic cells, was seen at 16 hours post injury and for tissue repair cells, due primarily to peak in fibroblasts, peaks were seen at 5 and 14 days post injury. Peaks in cells within PTG correlate with peaks in peritoneal reaction volumes seen at 1 day and 5 to 7 days and peak in peritoneal cell concentration at 5 days, demonstrating that cellular presence is distributed throughout injury and underlying/surrounding tissue. In addition peaks in inflammatory and subsequently repair tissue cells precede peaks in fibrinous and fibrous tissue generation volumes respectively. Hence maximum presence of inflammatory cells appears to stimulate fibrin deposition and maximum presence of tissue repair cells appears to lead to collagen deposition. However it was not until few inflammatory cells were present within PTG that maximum peritoneal damage was seen.

Maximum inflammatory cell densities within UTG occurred at 16 hours following injury and between 3 to 5 days for tissue repair cells. Peak in inflammatory cell densities again preceded the peak in fibrinous tissue generation and peak in uterine horn damage. Thus inflammatory cells are infiltrating into damaged tissues and removing such tissue by phagocytosis with tissue generated to replace removed tissue. Peaks in inflammatory cells also correspond to peaks in uterine horn haemorrhage.

With regard to number totals within PTG and UTG, peaks in inflammatory cells occur at 1 days and 5 days for tissue repair cells. Hence tissue generated by either the peritoneum or uterine horn in response to abrasion injury and treated with novel fibrin sealant demonstrated the same cellular responses. The distinction between inflammatory and tissue repair cells also highlights the controlled nature of wound healing.

Differences in number densities of total cells, eosinophilic cells, macrophages and fibroblasts were seen between PTG and UTG. It has been highlighted that although the peritoneum and uterine horn undergo the same injury process, the peritoneum sustains a greater extent of underlying and surrounding damage. This greater damage results in greater tissue ischaemia and necrosis requiring additional inflammatory cells to clear this damaged tissue, which is precisely the case with increased densities of inflammatory cells within PTG compared to UTG. Since the uterine horn has less damage to be cleared before tissue repair can be successfully initiated, tissue repair cells should be present in uterine horn tissue earlier than peritoneal tissue, which is again what was observed during this study.

Macrophages were the predominant cell type within fibrin sealant associated cell concentration at all time points investigated (3 to 7 days). At 3 and 7 days, eosinophilic cells were the second most predominant followed by fibroblasts whereas, at 5 days, this order was reversed. This change was due to a drop in number density of eosinophilic cells compared to fibroblasts, with the reason for this being the reduction in inflammatory response. At 5 days post injury there was also a significant drop in total number density however little difference is seen for number totals. Therefore numbers of cells present

varied little from 3 to 7 days post injury, but an increase in volume of fibrin sealant associated cell concentration occurred at 5 days. This could be due to increased cell sizes, due to active phagocytosis and/or increased biochemical processes, with the outcome becoming decreased fibrin sealant volume.

Conclusions

i. The normal cellular progression of adhesion-free healing was demonstrated with novel fibrin sealant.
ii. Inflammatory cells (eosinophilic cells, basophilic cells, macrophages) were the predominant cell types present in tissue generated from injured peritoneum and uterine horn treated with novel fibrin sealant for the first day following injury.
iii. Tissue repair cells (fibroblasts, macrophages) were the predominant cell types present in tissue generated from injured peritoneum and uterine horn treated with novel fibrin sealant from 3 days onwards following injury.
iv. Inflammatory cells were associated with fibrinous tissue generation and tissue repair cells with fibrous tissue generation.
v. Fibrin sealant associated cell concentration is an immune response to the presence of a foreign protein within the peritoneal cavity. Cells present were predominately macrophages, followed by eosinophilic cells and fibroblasts.
vi. It is hypothesised that macrophages and eosinophilic cells attracted fibroblasts to the site of novel fibrin sealant, aiding wound healing.

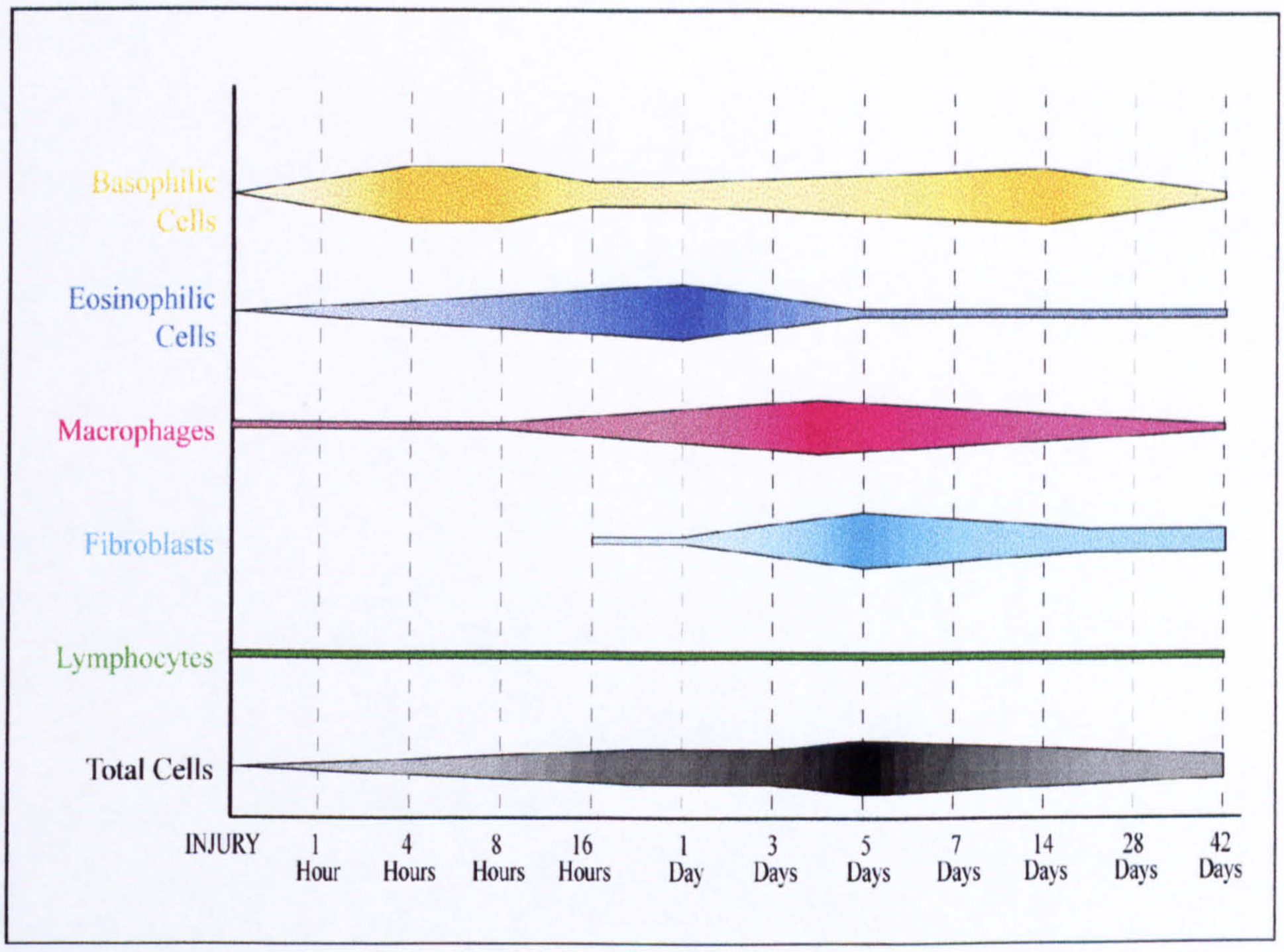


Figure 4.13: Summary diagram of cellular presence in injury tissue.

4.7 Biochemical analyses

As for Pathogenesis of PSAs study, blood and peritoneal fluid samples were taken throughout the surgical and termination procedures to gain understanding of cellular and biochemical changes during PSA prevention with novel fibrin sealant.

4.7.1 Full and Differential Blood Counts

Three blood samples were taken during this study: blood sample 1 taken pre-operatively (control); blood sample 2 taken immediately following laparotomy (laparotomy control); and blood sample 3 taken prior to termination (experimental sample). Total numbers of RBCs, platelets and WBCs were determined using an auto-analyser whereas WBC differential counts were performed by manual methods (refer to Chapter 2).

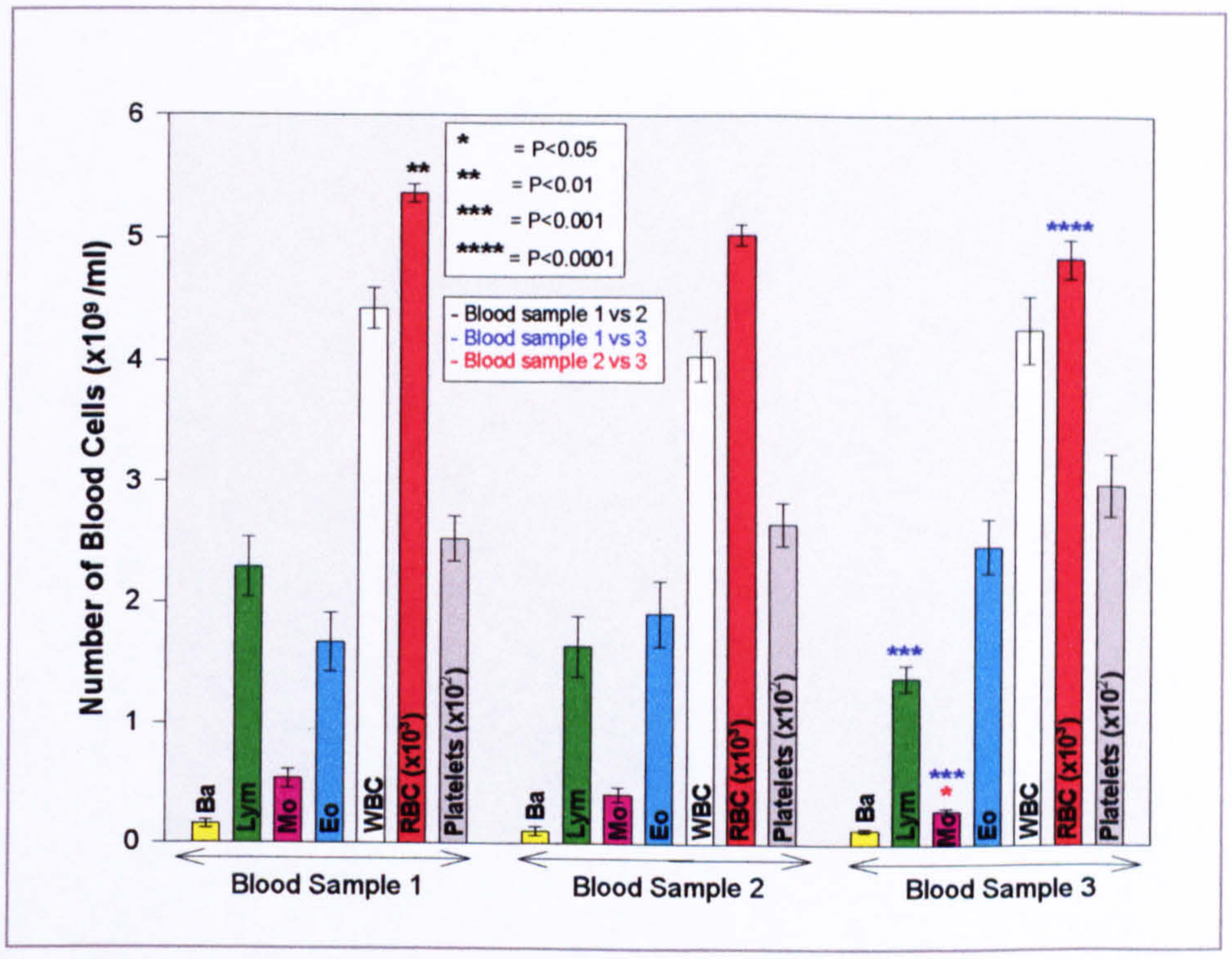
4.7.1.1 Results

Overall mean number of RBCs for control blood sample 1 was 5.60 $x10^{12}$/l, with 253 $x10^{9}$/l platelets, 4.44 $x10^{9}$/l WBCs, 0.15 $x10^{9}$/l basophilic cells, 2.28 $x10^{9}$/l lymphocytes, 0.54 $x10^{9}$/l monocytes and 1.68 $x10^{9}$/l eosinophilic cells (Graph 4.21; Appendix IV Table 4.13).

Laparotomy control blood sample 2 demonstrated mean numbers of 5.16 $x10^{12}$/l RBCs, 266 $x10^{9}$/l platelets, 4.06 $x10^{9}$/l WBCs, with 0.10 $x10^{9}$/l basophilic cells, 1.64 $x10^{9}$/l lymphocytes, 0.42 $x10^{9}$/l monocytes and 1.91 $x10^{9}$/l eosinophilic cells (Graph 4.21; Appendix IV Table 4.13).

Mean numbers of blood cells present in experimental blood sample 3 were 4.83 $x10^{12}$/l RBCs, 285 $x10^{9}$/l platelets, 4.26 $x10^{9}$/l WBCs, with 0.12 $x10^{9}$/l basophilic cells, 1.39 $x10^{9}$/l lymphocytes, 0.28 $x10^{9}$/l monocytes and 2.47 $x10^{9}$/l eosinophilic cells (Graph 4.21; Appendix IV Table 4.13).

T test analysis demonstrated a significant difference ($P=0.034$) in mean numbers of RBCs between control and laparotomy control samples (blood samples 1 and 2). Comparison between control (blood sample 1) and experimental (blood sample 3) samples revealed significant differences in mean numbers of lymphocytes ($P=0.0004$), macrophages (0.0003) and RBCs ($P<0.0001$). A significant difference ($P=0.037$) was seen in mean monocyte number between laparotomy control (blood sample 2) and experimental sample (blood sample 3) (Graph 4.21; Appendix IV Table 4.14).



Graph 4.21: Mean numbers of full and differential blood cell counts for all blood samples together with SEMs and statistical differences.

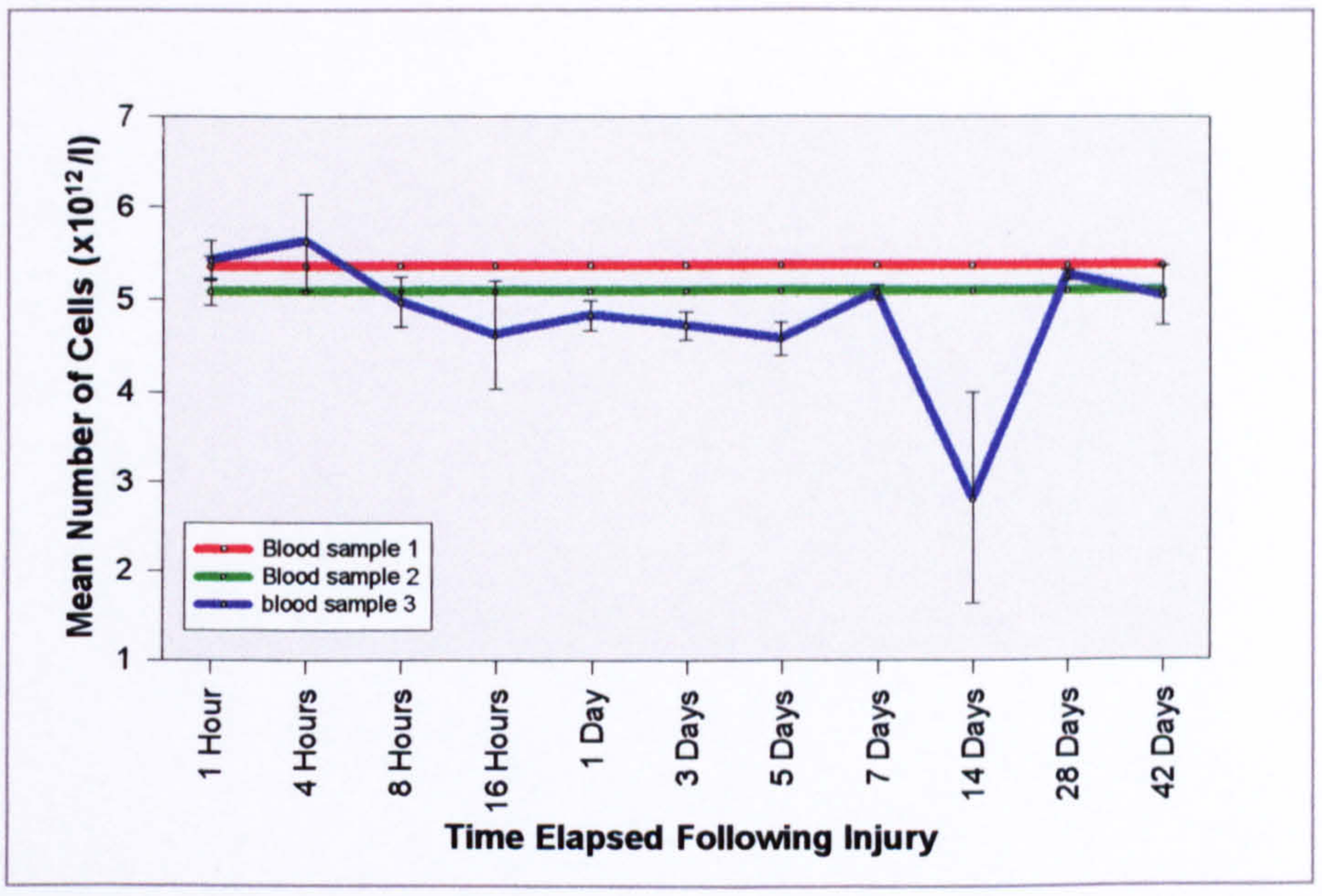
Note:

Ba =	basophilic cells;	Lym =	lymphocytes;
Eo =	eosinophilic cells;	Mo =	monocytes.

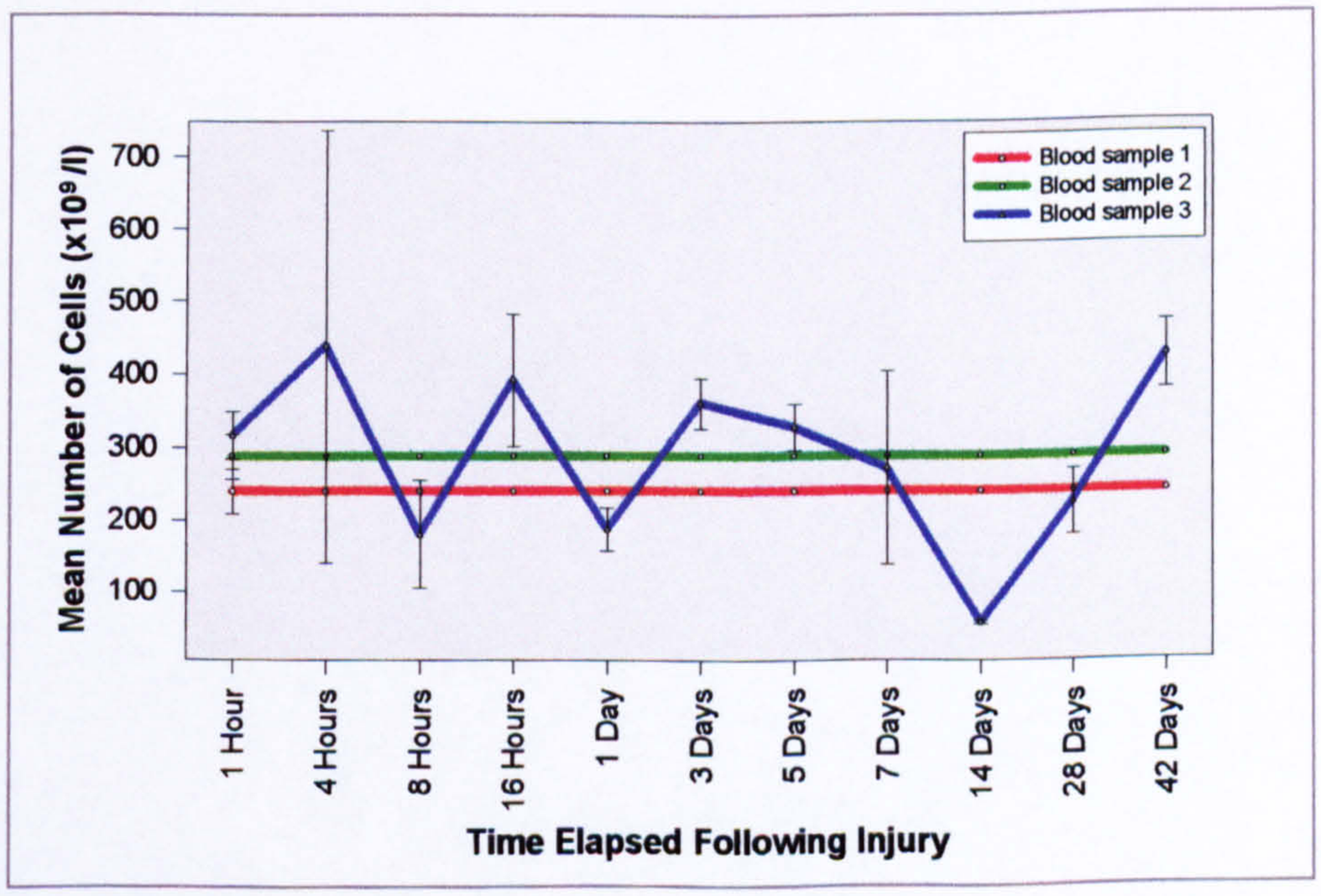
Mean numbers of both RBCs and platelets at time points following injury for experimental sample 3 demonstrated similar levels to controls and laparotomy control throughout this study, except for 14 day time point at which there was a decrease for both cell types (Graphs 4.22 and 4.23; Appendix IV Table 4.15).

Mean numbers of WBCs in experimental sample 3 fluctuated throughout the study (Graph 4.24; Appendix IV Table 4.15). An increase was seen between 1 and 8 hours after which numbers returned to control levels. A slight decrease occurred at 3 days, which was followed by an increase at 5 days and then subsequent decrease between 7 and 14 days. By 28 and 42 days mean numbers were within normal control levels.

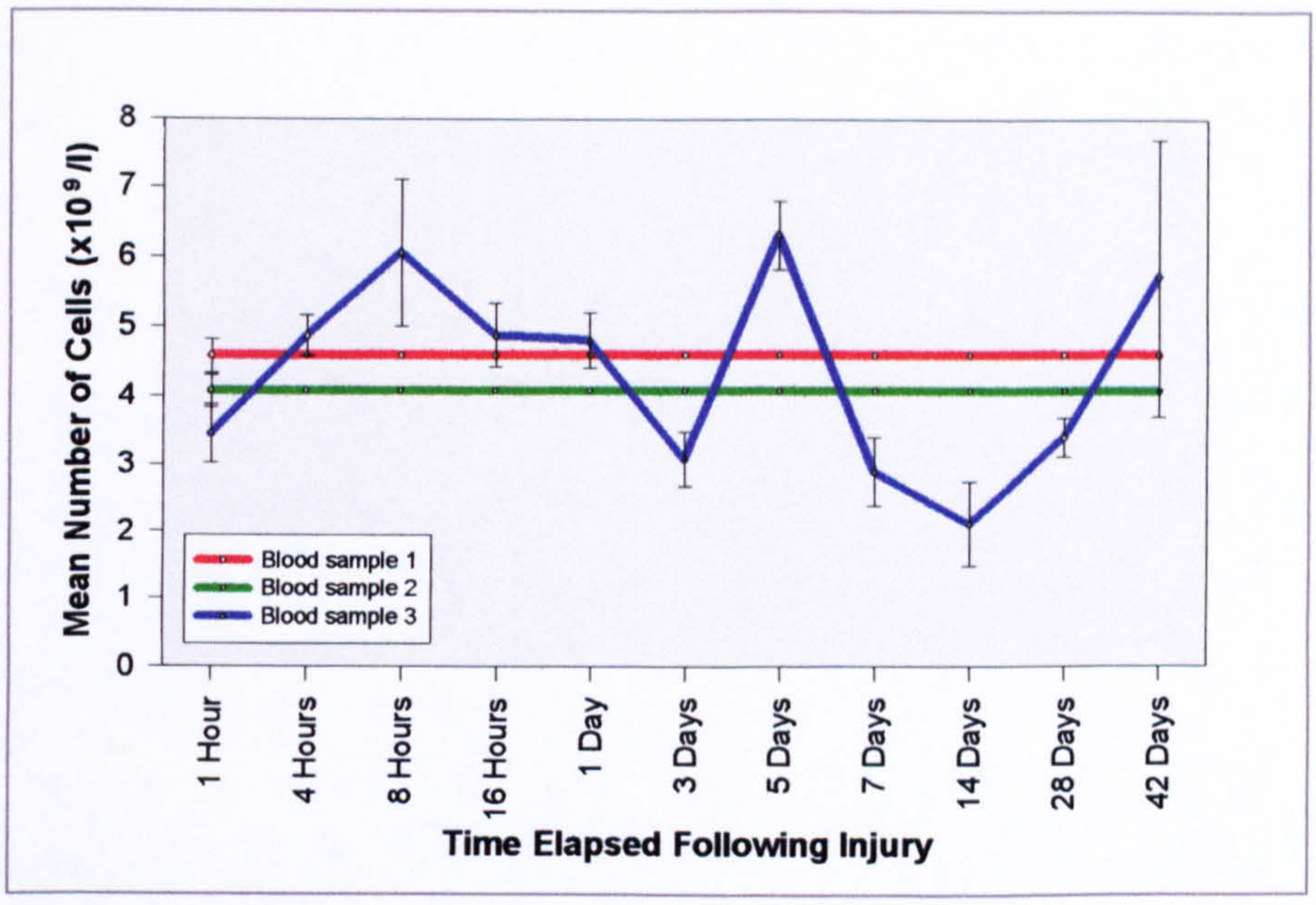
Basophilic cells demonstrated a trend to increase in number from 1 hour to 1 day with a sharp decrease to 3 days (Graph 4.25; Appendix IV Table 4.15). Mean numbers remained below controls from 3 to 14 days, with an increase seen to 42 days post injury.



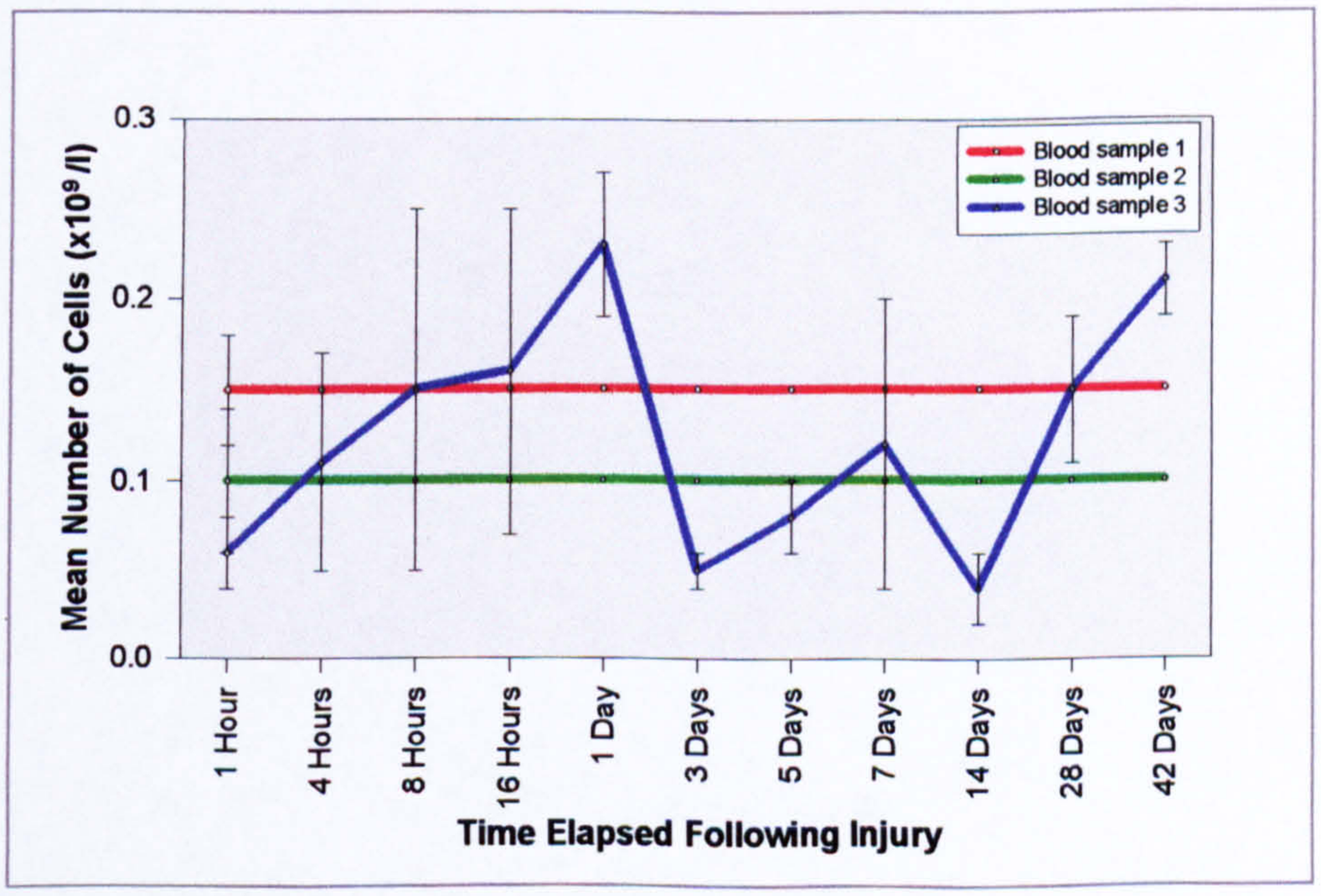
Graph 4.22: Mean numbers of RBCs for all blood samples together with SEMs.



Graph 4.23: Mean numbers of platelets for all blood samples together with SEMs.



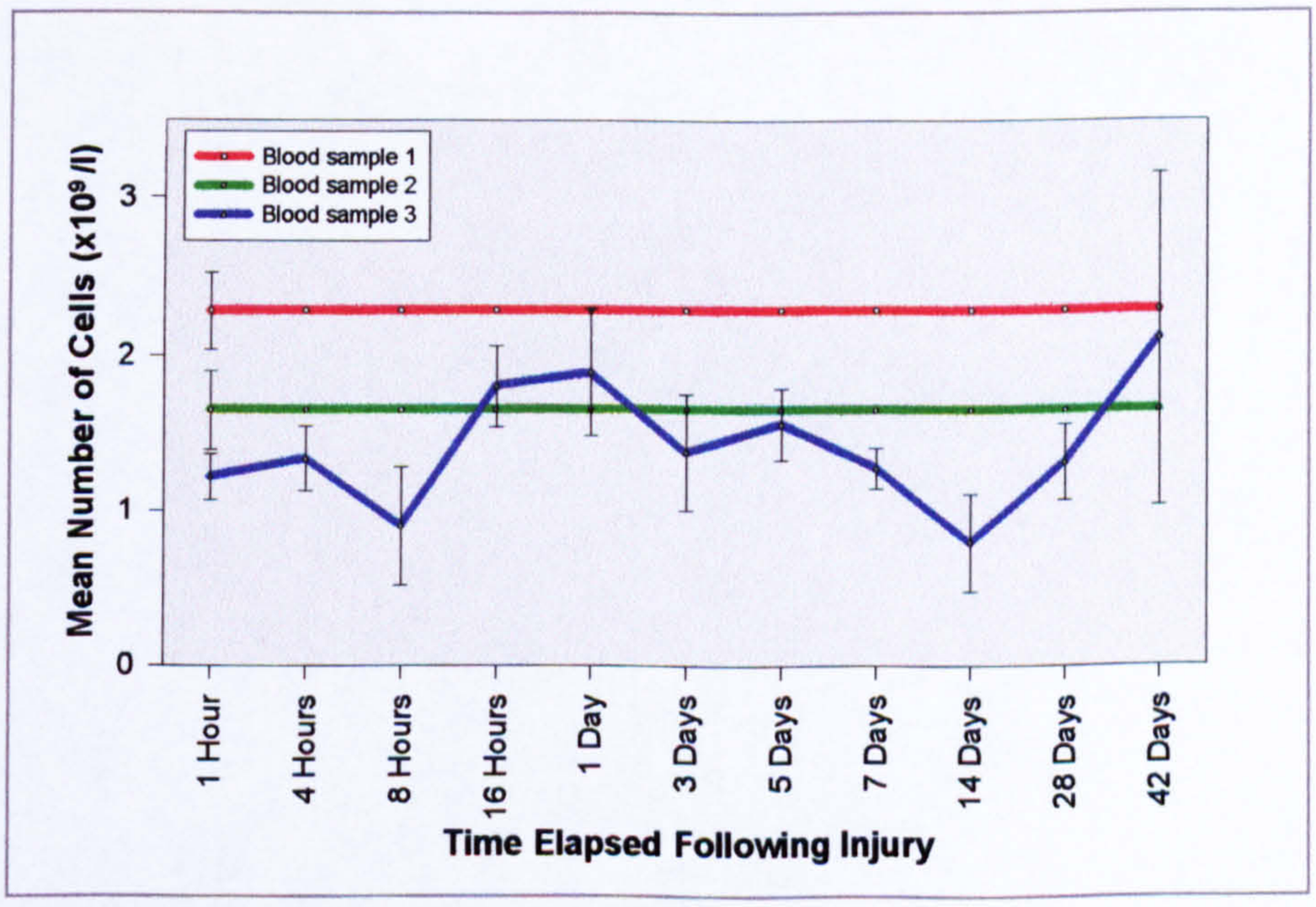
Graph 4.24: Mean numbers of WBCs for all blood samples together with SEMs.



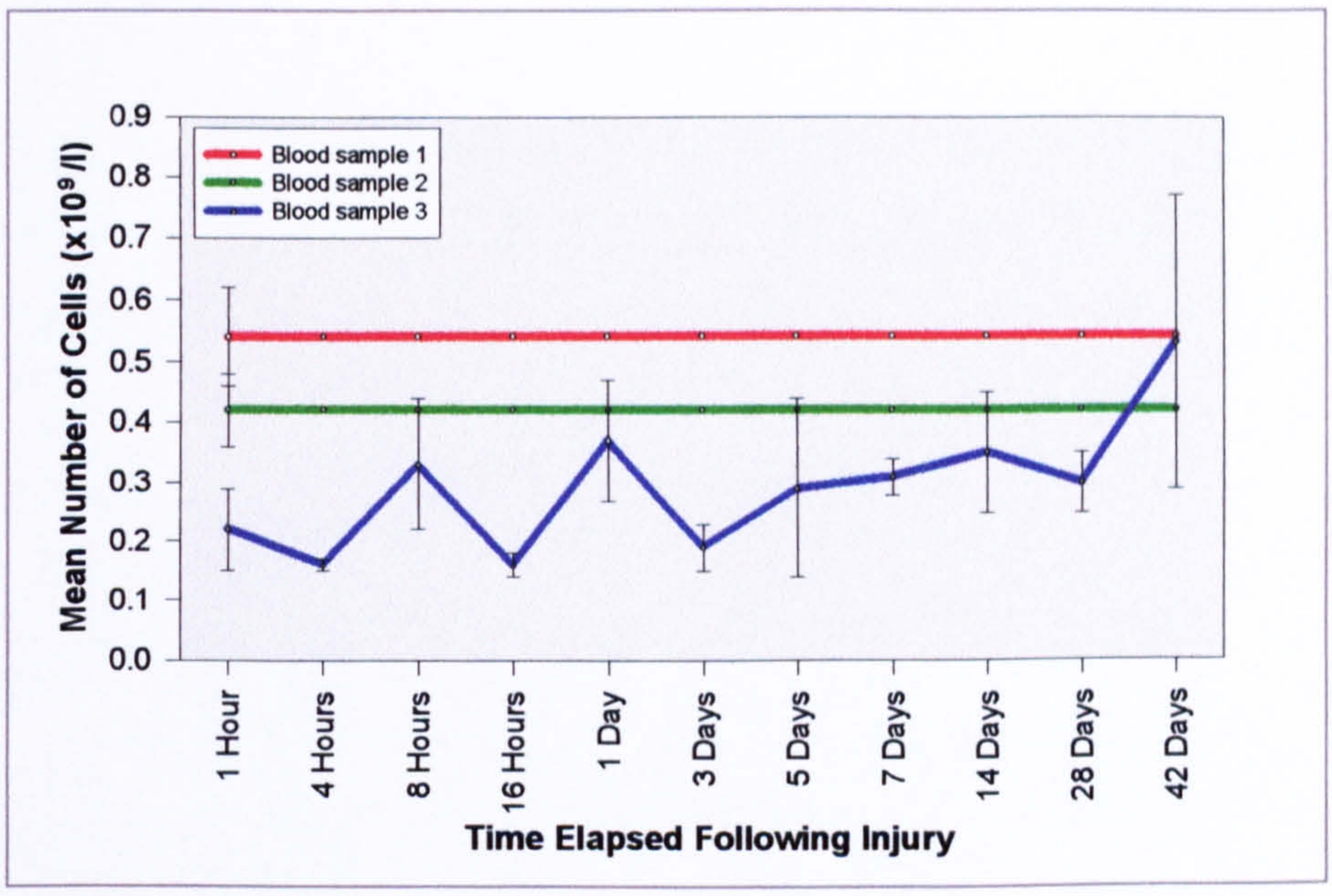
Graph 4.25: Mean numbers of basophilic cells for all blood samples together with SEMs.

Mean numbers of lymphocytes in experimental sample 3 were generally slightly below control levels throughout most of this study, with only mild fluctuations demonstrated (Graph 4.26; Appendix IV Table 4.15). Little change was seen from 1 to 8 hours after which there was a trend to increase. Mean numbers showed a gradually decrease from 1 to 14 days with a subsequent increase to control levels by the completion of this study. In a similar manner monocytes were slightly below control levels throughout this study until 42 days post injury (Graph 4.27; Appendix IV Table 4.15). There were no real trends in mean monocyte number.

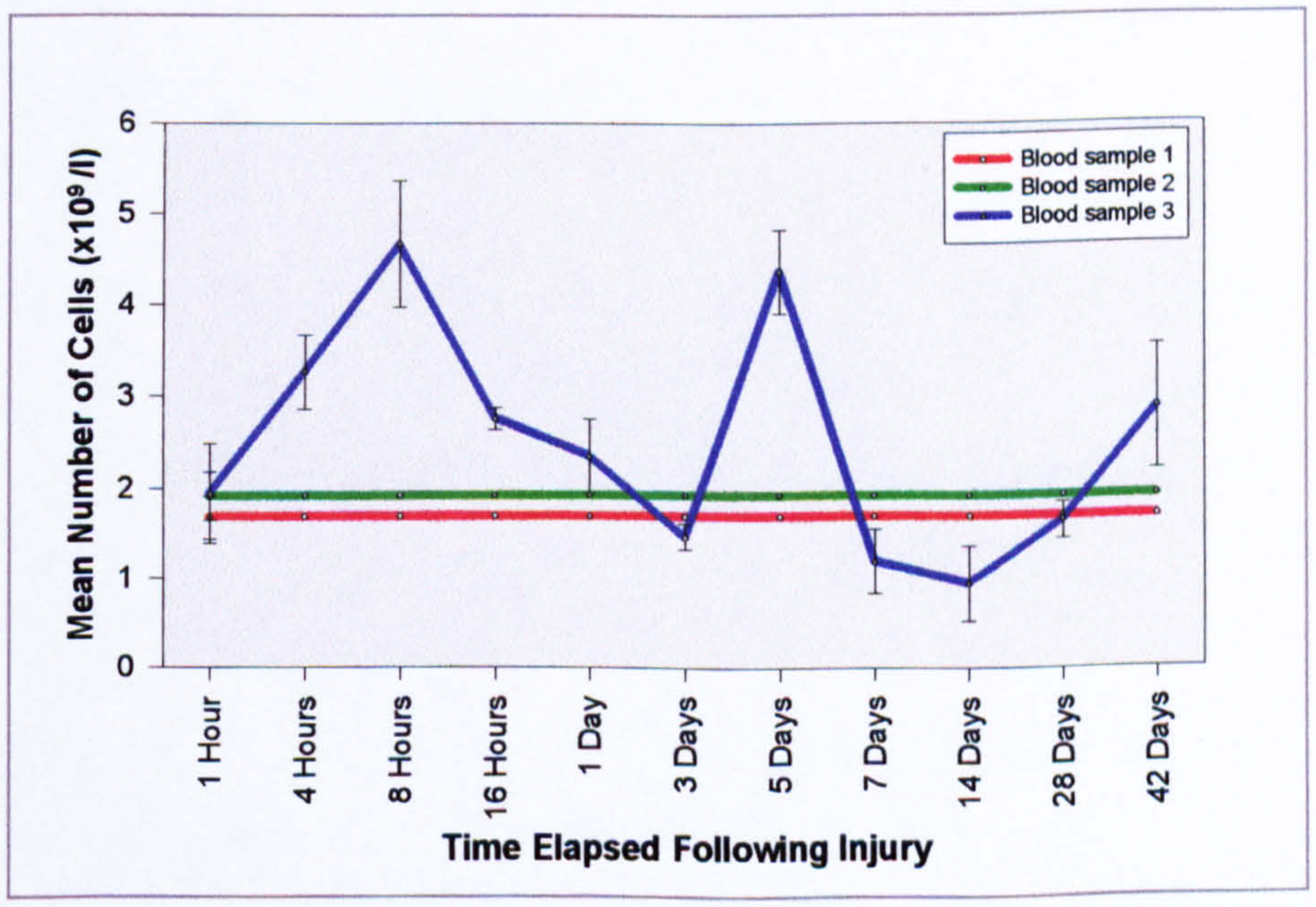
In experimental sample 3 eosinophilic cells demonstrated several trends in mean number from 1 hour to 42 days following injury (Graph 4.28; Appendix IV Table 4.15). From 1 hour eosinophilic cells rapidly increased to peak at 8 hours after which numbers steady decreased to control levels by 3 days. Another peak was seen at 5 days with a rapid decrease to 7 days with numbers returning to control values by 14 to 28 days post injury.



Graph 4.26: Mean numbers of lymphocytes for all blood samples together with SEMs.



Graph 4.27: Mean numbers of monocytes for all blood samples together with SEMs.



Graph 4.28: Mean numbers of eosinophilic cells for all blood samples together with SEMs.

4.7.1.2 Discussion

As discussed in Section 3.7.1.2 systemic levels of rabbit cells show great variability and are effected by venesection of volumes of 10 to 20ml and above (Figure 4.14). Therefore these effects need to be considered when interpreting the results obtained in this study.

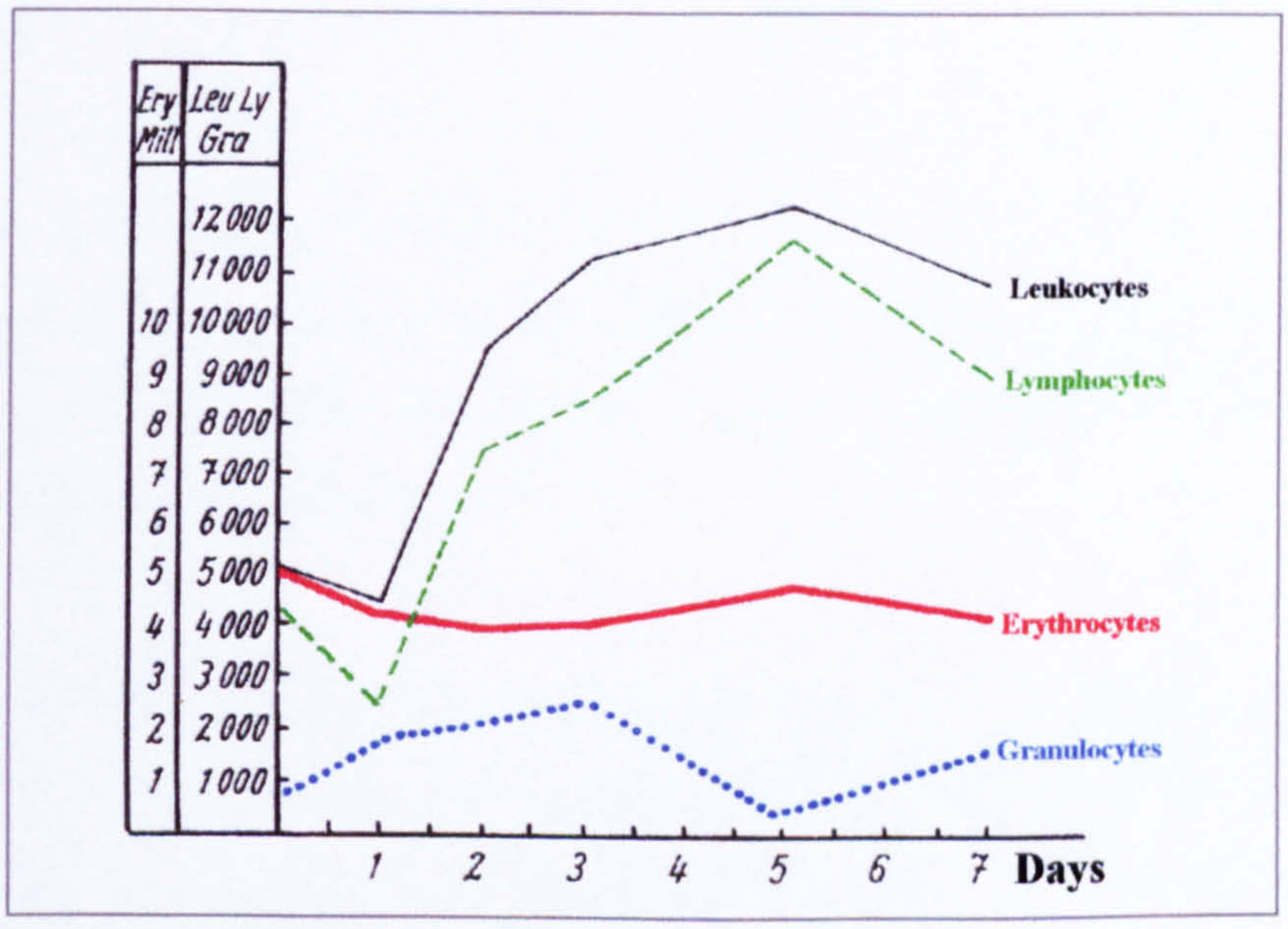


Figure 4.14: Rabbits response to venesection (20ml)
(Taken from Schermer, 1967).

All blood cell types demonstrated mean numbers within normal ranges for all blood samples. Mean numbers of WBCs and lymphocytes were at the very low end of the normal values reported by Sanderson & Phillips (1981) (Appendix III Table 3.16) however considerable variations in WBC count are well recognised (Schermer, 1967).

Comparison of pre-operative control (sample 1) with laparotomy control (sample 2) demonstrated only one significant difference. The laparotomy control showed significantly lower mean number of RBCs compared to pre-operative controls. This is most probably due to plasma volume increasing in response to the removal of the first blood sample, diluting the blood cells. Support for this hypothesis is seen by lower, although not significantly ($P>0.05$) mean numbers of WBCs in the laparotomy control. This difference between RBCs and WBCs are attributed to the easier and quicker recruitment of WBCs into the circulatory system.

Mean numbers of both RBCs and platelets in experimental blood sample 3 showed little variation over the duration of this study. Hence neither venesection, prevention with novel fibrin sealant nor wound healing had any substantial effect on the cellular profiles of both these cell types.

WBCs demonstrated an increase in mean number from 1 hour and levels remained slightly above normal until 1 day. This does not follow the pattern for venesection effected blood, indicating that either the introduction of novel fibrin sealant and/or wound healing had a rapid response on WBCs causing their increased circulation. The consequences of this appear to be extended throughout most of this study with the effect of blood collection undetermined.

Mean numbers of basophilic cells at each time point demonstrated large variations probably due to their relatively low numbers. Changes in the number of these cells in circulation appears to be influenced by venesection rather than the presence of novel fibrin sealant and/or wound healing.

Lymphocytes demonstrated lower levels at most time points compared to controls with few trends seen. Hence the expected effects of blood collection were absent, either due to no effects occurring or that these effects are masked by lymphocyte recruitment and migration out of circulation in response to novel fibrin sealant and/or wound healing. From the results in this study it appears that either *in vivo* application of novel fibrin sealant and/or wound healing lowered the mean number of systemic lymphocytes.

Levels of systemic monocytes were similar to lymphocytes in that they were generally lower compared to controls and showed few fluctuations. Mild peaks in mean number were seen at 8 hours and 1 day, which could be attributed to blood collection, with a further peak at 42 days returning levels to normal values. Hence treatment with novel fibrin and/or wound healing had the general effect of lowering the level of circulating monocytes.

Novel fibrin sealant and/or wound healing had the greatest effect on circulating eosinophilic cells. During the course of this study two peaks were seen in mean number, at 8 hours and 5 days. From comparison with venesection results, these peaks are most likely to be due to the presence of novel fibrin sealant or wound healing. Eosinophils are been implemented in the early stages PSA development (Milligan & Raftery, 1974) with an anti-histamine and therefore anti-inflammatory activity. It would appear that treatment with novel fibrin sealant and/or wound healing resulted in increased release of these cells into circulation, which may play some role in PSA prevention.

Conclusions

i. Care needs to be taken in interpreting full and differential counts obtained in this study due to the unreliability of rabbit blood factors, effects of venesection and small sample size for the differential counts.
ii. Blood cell types demonstrated mean numbers within normal ranges for all blood samples.
iii. Laparotomy control showed significantly lower mean number of RBCs compared to pre-operative controls due to plasma infiltration/dilution in response to the initial venesection.
iv. Mean numbers of both RBCs and platelets showed little variation over the duration of this study.
v. Introduction of novel fibrin sealant and/or wound healing had a rapid response on WBCs resulting in their increased circulation. The effect was extended throughout most of this study with the outcome of blood collection undetermined.
vi. Mean numbers of basophilic cells at each time point demonstrated large variations probably due to their relatively low numbers with changes attributed to venesection.
vii. Lymphocytes demonstrated lower levels at most time points compared to controls, with few trends seen, probably due to novel fibrin sealant treatment and/or wound healing.
viii. Levels of systemic monocytes were similar to lymphocytes in that they were generally lower compared to controls and showed few fluctuations. This is attributed to treatment with novel fibrin and/or wound healing.

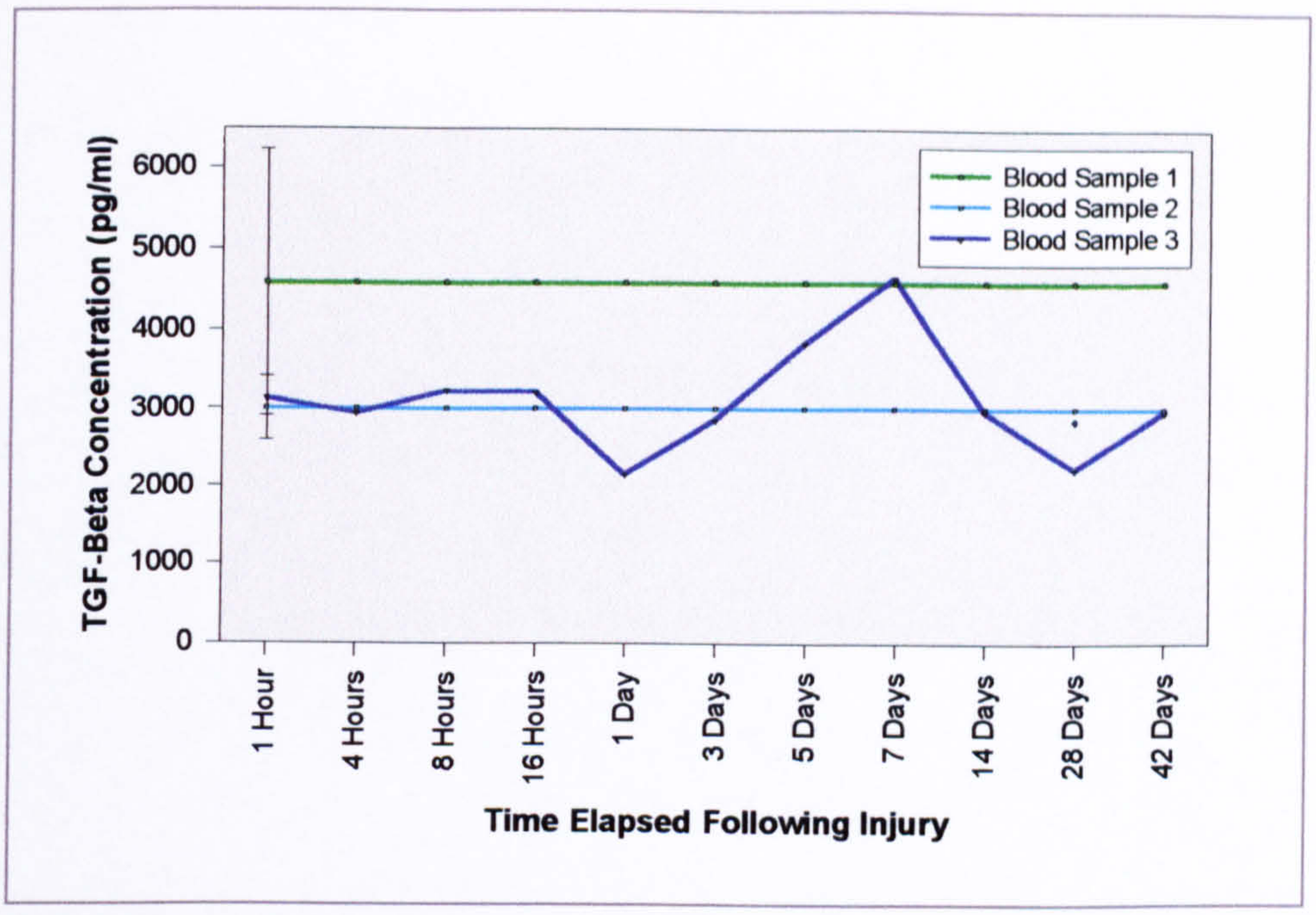
ix. Two peaks were seen in eosinophilic cell mean number, at 8 hours and 5 days, most likely to be due to the presence of novel fibrin sealant and/or wound healing resulting in the increased release of these cells into circulation, which may play some role in PSA prevention.

4.7.2 TGF-β Concentrations

Only one of the ELISA kits investigated was found to detect rabbit cytokines accurately and reproducibly, the R&D Systems TGF-β ELISA. Due to time limitations only one plasma sample from each time point and one undiluted peritoneal fluid sample from 3 to 28 days, together with the peritoneal sample from the adhered case and controls were assayed (refer to Chapter 2 for methodology).

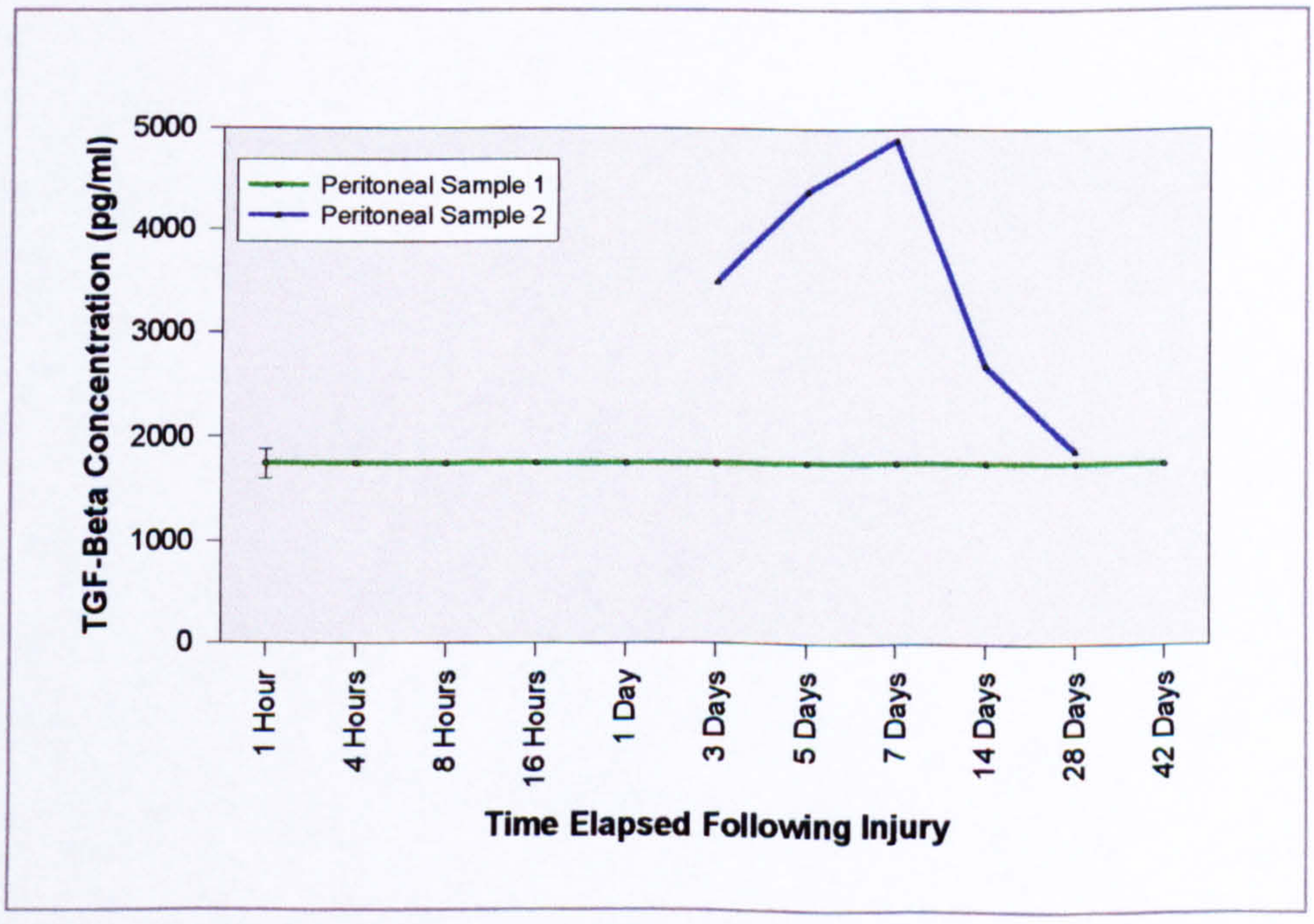
4.7.2.1 Results

TGF-β concentrations within pre-operative control blood sample 1, post laparotomy sample 2 and experimental samples 3 were all similar (Graph 4.29; Appendix IV Table 4.16). Coefficient of variation was calculated to be 36% for control samples 1 and 13% for control samples 2. Overall experimental samples 3 demonstrated similar concentrations from 1 hour to 42 days however there was a peak at 7 days post injury.



Graph 4.29: TGF-β concentrations present in blood samples.

TGF-β concentrations were detected in all both control and experimental peritoneal fluid samples (Graph 4.30; Appendix IV Table 4.16). Control peritoneal fluid samples 1 demonstrated 8% variance . TGF-β levels in experimental samples 3 varied throughout wound healing with maximum concentration seen at 7 days and a decline thereafter to control values at 28 days.



Graph 4.30: TGF-β concentration present in peritoneal fluid samples.

4.7.2.2 Discussion

Due to the very minimal numbers of samples assayed only preliminary results have been obtained and therefore should be cautiously interpreted.

All plasma and peritoneal fluid samples demonstrated levels of TGF-β. Typically TGF-β is present in serum at low levels. Donor serum and plasma samples from apparently healthy individuals activated and assayed by R&D Systems demonstrated TGF-β concentration of 48.6ng/ml and 2.26ng/ml respectively. Since our study comprised of rabbit samples direct comparisons between resting levels of TGF-β can not be made.

TGF-β is produced by many cell types including platelets, fibroblasts, myocytes, astrocytes and epithelial cells. This cytokine is associated with wound healing and is present at injury sites due to the presence of degranulating platelets. It causes cellular chemotaxis, protein matrix synthesis and further secretion of factors which combine with TGF-β to mediate angiogenesis and fibrosis.

TGF-β concentrations within experimental samples were all within control levels and hence it appears that treatment of injury sites with novel fibrin sealant does not have any effect on systemic levels of TGF-β. However variations were seen with experimental peritoneal samples compared to controls with peak concentration at 7 days post injury, which follows the peak in fibroblast cell numbers. Hence it possible that fibroblasts present within injury tissue are secreting TGF-β which is in turn mediating angiogenesis and tissue fibrosis.

Conclusions

i. Biochemical control of wound healing by TGF-β may be occurring locally within peritoneal fluid.
ii. Further work is required to substantiate or rebut this theory.

4.8 Conclusions

Using a definitive, reproducible PSA inducing model, treatment with novel fibrin sealant prevented PSA formation in 98% of cases. This is by far the best reported PSA reduction with a fibrin sealant. The additional benefits of quick and easy preparation and application with minimal human input and elimination of potential viral and antigenicity problems, make novel fibrin sealant an excellent PSA preventative agent.

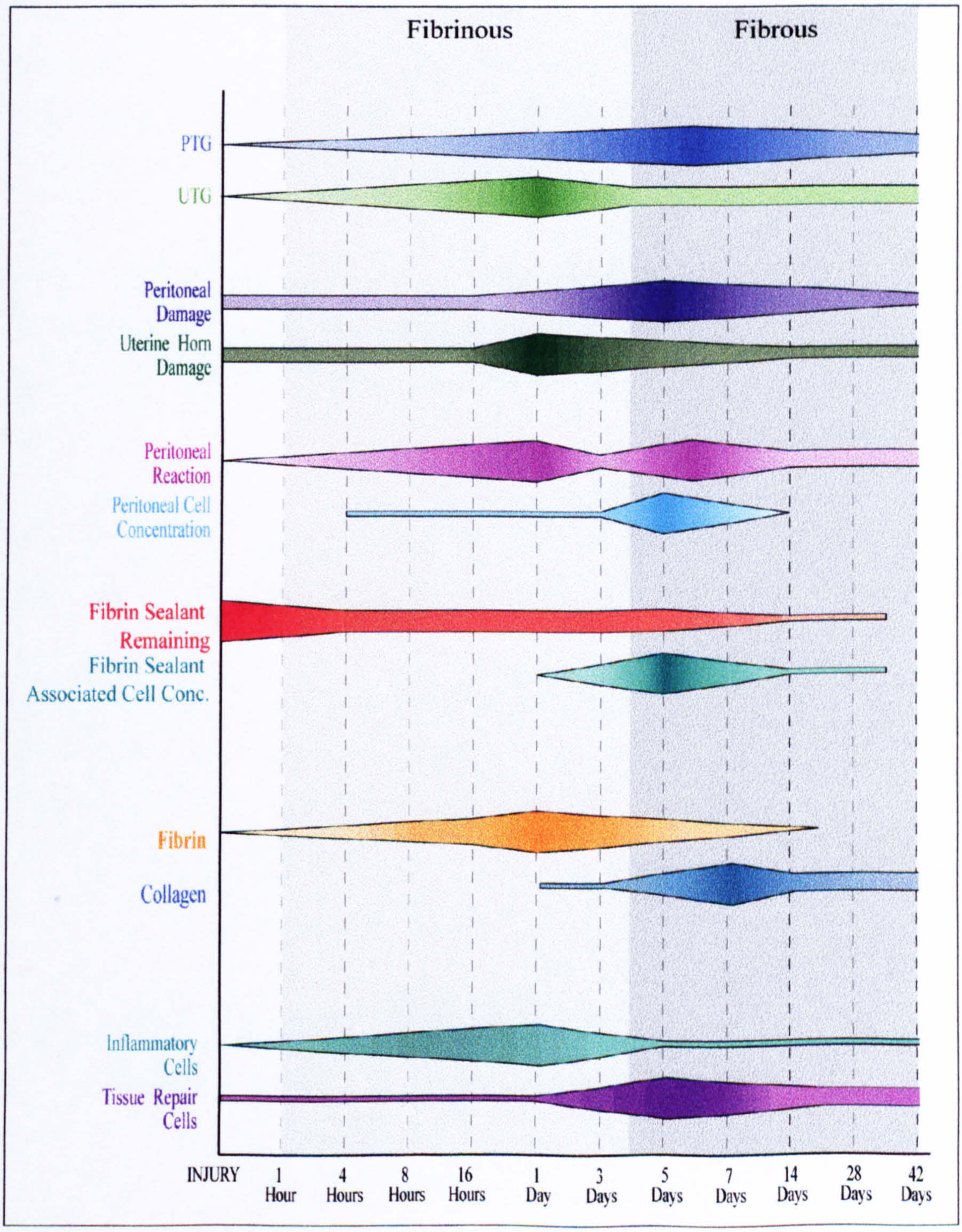


Figure 4.15: Summary of quantitative results.

Treatment with novel fibrin sealant does not interfere with the normal progression of wound healing with regard to physiological, cellular and biochemical mechanisms (Figure 4.15 and 4.16). Following injury fibrin exudate was released with the formation of repair tissue discretely located on each injury site but not joining the two. This repair tissue was fibrinous up to 3 days post injury, with maximum volume seen at 1 day, and fibrous from 5 days onwards, with maximum fibrous volume at 7 days.

Only damage from the original abrasion injury was present for the first 16 hours after which there was an increase corresponding to phagocytosis and clearance of damaged surrounding and underlying tissue by phagocytes. Following clearance damaged tissue was replaced with new repair tissue.

During the first day following injury inflammatory cells (eosinophilic cells, basophilic cells, macrophages) were the predominant cell types present within injury tissue. Whereas tissue repair cells (fibroblasts, macrophages) were predominant from 3 days onwards.

Local mediation of angiogenesis and fibrosis by TGF-β within peritoneal fluid may also be occurring. Further analyses are required to substantiate and expand on this hypothesis.

Hence adhesion-free healing of mechanically injured tissues by novel fibrin sealant can be classified into two phases (Figure 4.16):

i. Tissue modelling: up to/including 1 day post injury
   Includes: Inflammatory response
   Fibrin deposition

ii. Tissue remodelling: from 3 days post injury and onwards
   Includes: Tissue repair
   Collagen deposition
   Biochemical modulation by TGF-β

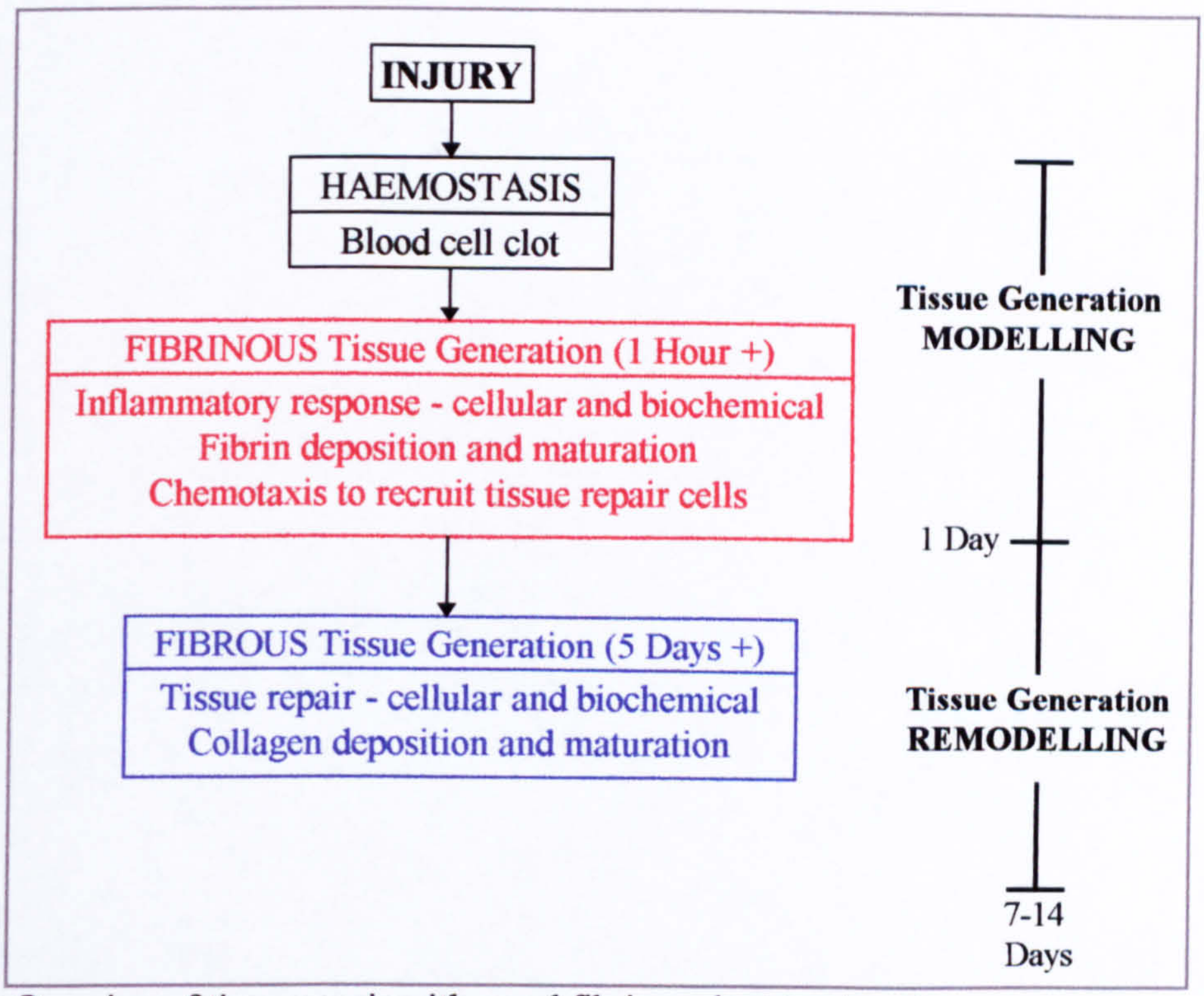


Figure 4.16: Overview of tissue repair with novel fibrin sealant treatment.

In vivo volume of novel fibrin sealant remained relatively constant for the first 5 days following application, after which there was a rapid loss, due to either phagocytosis and/or fibrinolysis. Therefore novel fibrin sealant was present during tissue generation modelling, fibrin deposition, and during the initial stages of tissue generation remodelling, collagen deposition. Novel fibrin sealant presence at injury site(s) during these stages is highly important and probably the major reason behind its PSA prevention success.

A cellular response to human novel fibrin sealant in this rabbit experimental model was evident from 3 to 7 days post injury, with maximum response seen at 5 days. This response primarily consisted of macrophages and eosinophilic cells. It is hypothesised that this is an immune mediated response to the presence of foreign protein within the peritoneum, with phagocytic cells attracted to phagocytose this foreign material. Such a response should be absent when novel fibrin sealant is used as intended, that is, as a truly autologous sealant.

5.0 Comparison of PSA Development and Prevention with Vivostat™ System Human Derived Fibrin Sealant

5.1 Introduction

Chapter 3 of this thesis describes the first study in which physiological, structural, cellular (local and systemic) and biochemical mechanisms of PSA development have been simultaneously quantitatively analysed. In order to prevent PSAs such knowledge is essential not only in targeting of preventative agents but also to understand their mechanisms of action to potentially improve their efficiency.

Many previous studies investigating (Vivostat™ system human derived fibrin sealant) novel fibrin sealant have demonstrated its ability to prevent PSAs. In order to understand its mechanisms of action, analogous analyses were performed to those investigating PSA development: quantitative analysis of physiological, structural, cellular (local and systemic) and biochemical mechanisms (refer to Chapter 4). To fully comprehend PSA prevention by novel fibrin sealant, direct comparisons need to be made between data from novel fibrin sealant PSA prevention and PSA development.

A fault of previous studies has been the lack of fair comparisons which could be made between control and treated cases and between studies investigating different treatments. In this study these problems are overcome by:

i. the use of an identical, definitive experimental model (standard and reproducible injury)
ii. identical sample time points
iii. strict quantitative analysis which is unbiased and precise.

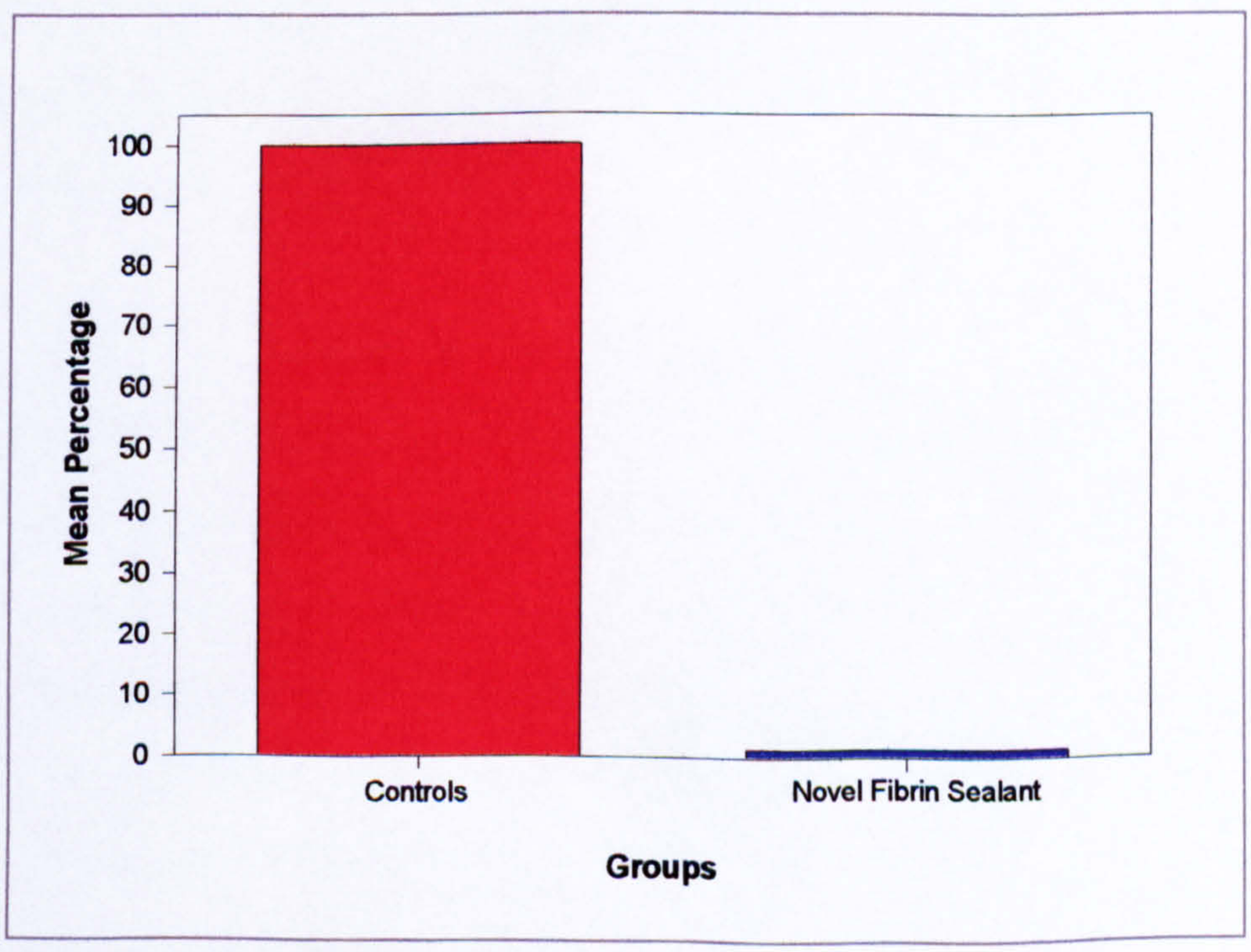
During this chapter data obtained from the PSA development study will be referred to as control data/cases and from the PSA prevention with novel fibrin sealant as treated data/cases.

5.2 Volumetric Analysis of Tissue Zones

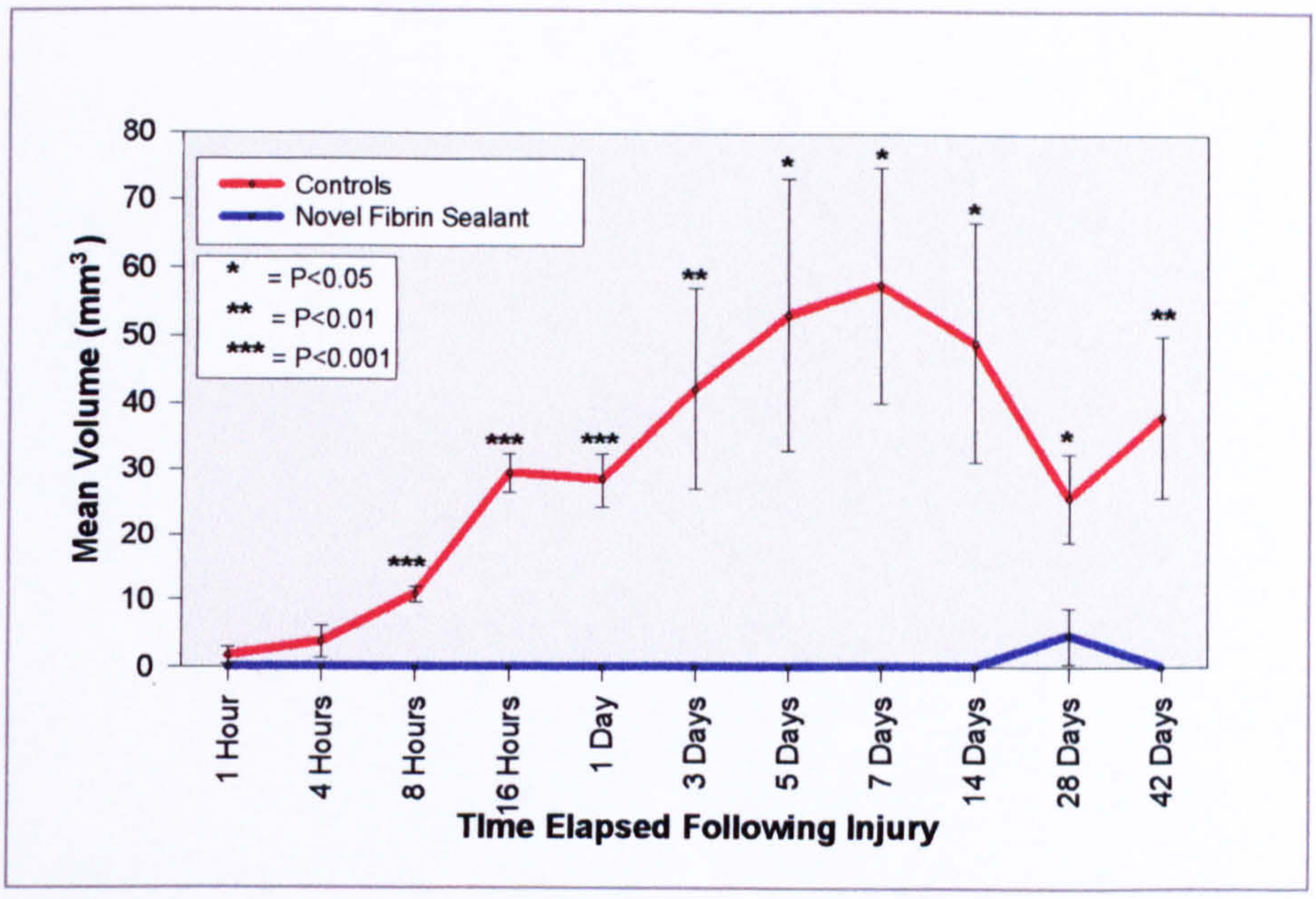
5.2.1 Results

Incidence of PSAs in the rabbit uterine horn abrasion model was 85% in the control group and 3% in the novel fibrin sealant group (from 8 hours following injury and onwards). There was a 96% reduction in the incidence of PSAs with the use of novel fibrin sealant. Mean volume of PSAs for control cases was 339.62mm^3 and 4.71mm^3 novel fibrin sealant cases. If we assume control cases have a percentage mean volume of 100% then novel fibrin sealant cases showed a percentage volume of 1.4% (Graph 5.1). Hence treatment with novel fibrin sealant produced a 98.6% decrease in volume of PSAs.

With regard to mean volume of PSA at each time point, all control groups from 1 hour post injury and onwards demonstrated PSAs whereas only one human derived fibrin sealant novel fibrin sealant case (28 day) demonstrated PSAs (Graph 5.2). All controls groups from 8 hours onwards showed significantly greater ($P<0.05$) mean volumes of PSAs compared to novel fibrin sealant treated groups (Appendix V Table 5.1).



Graph 5.1: Mean PSA percentages.

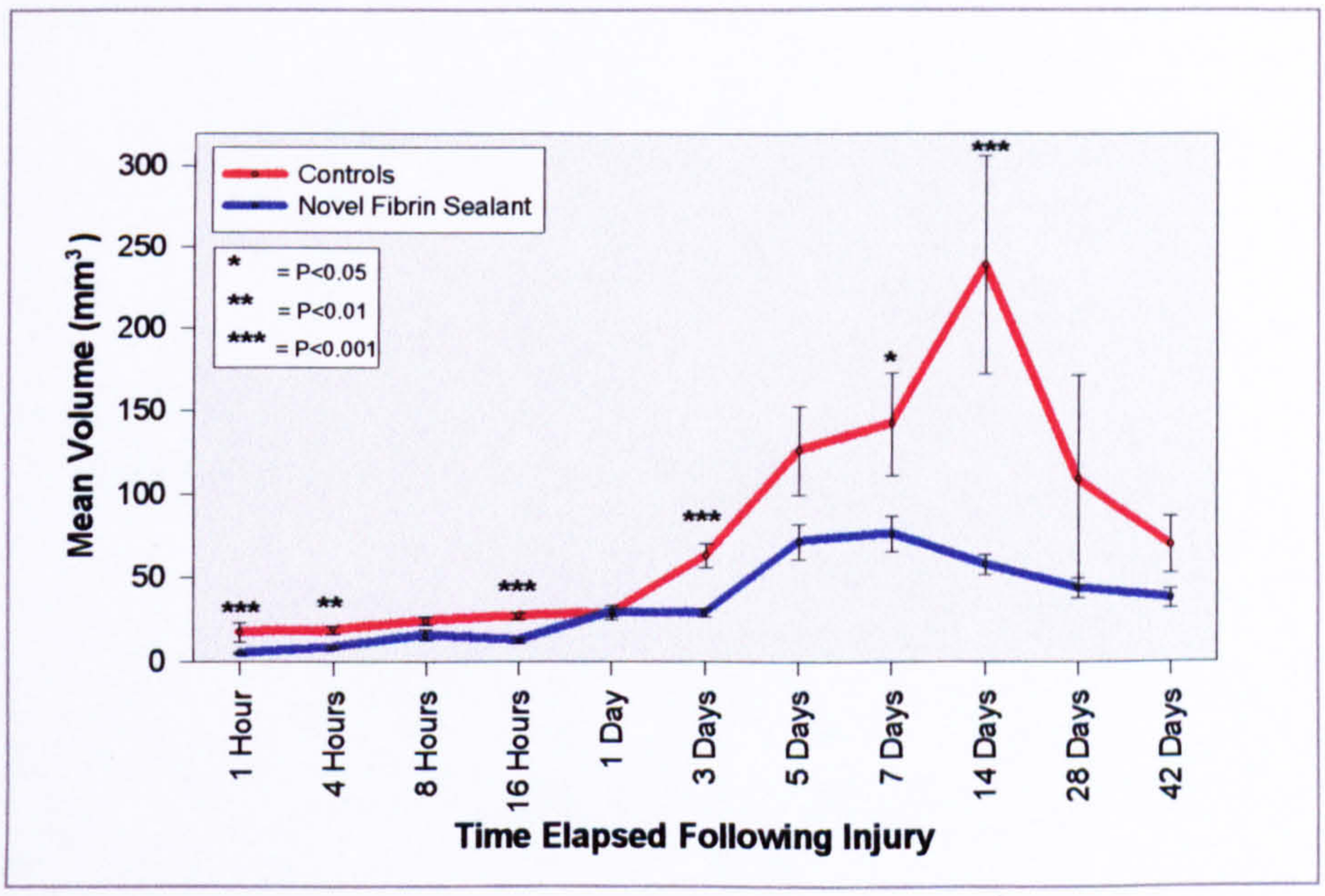


Graph 5.2: Mean PSA volumes
together with SEMs and statistical differences.

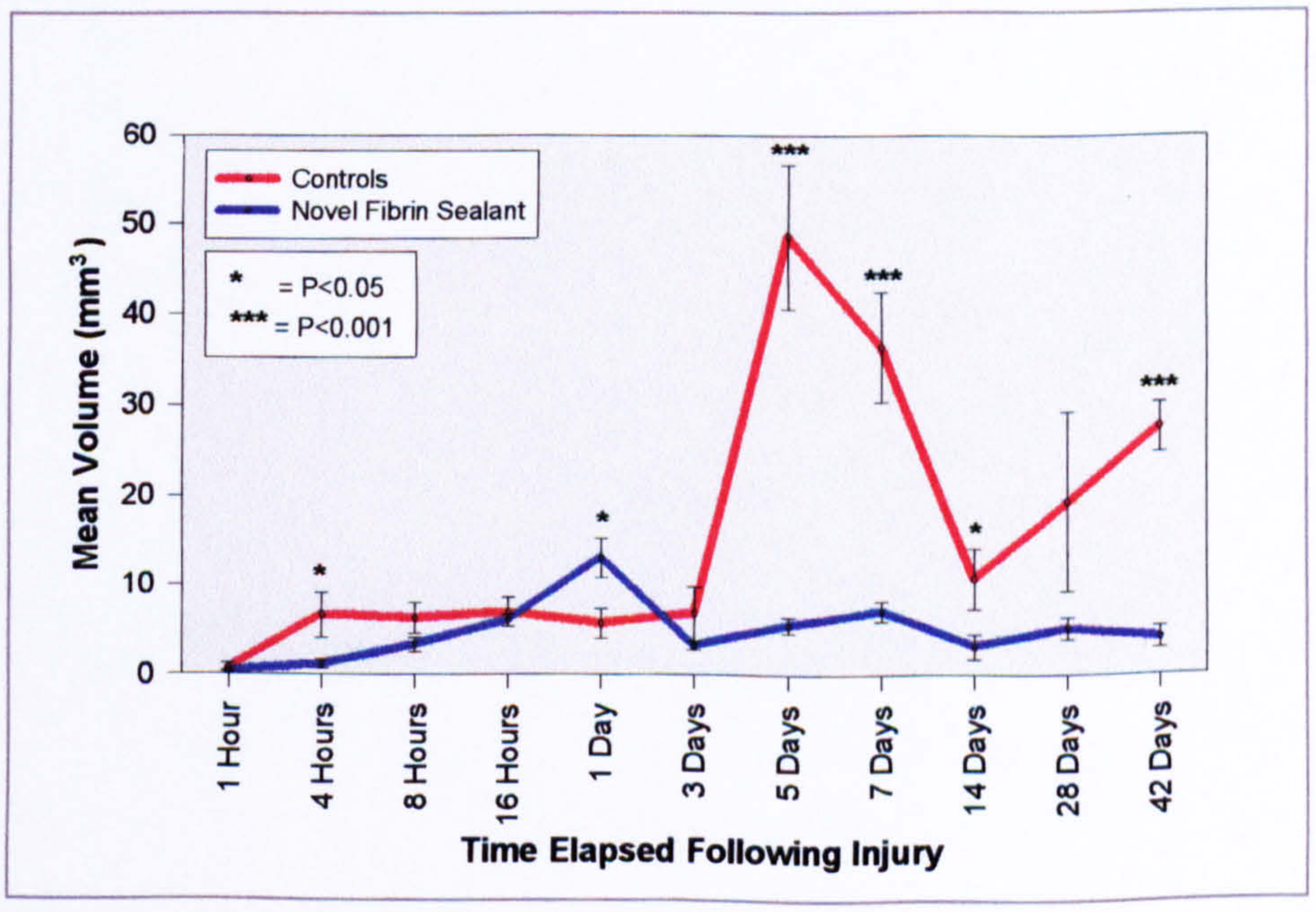
Control and novel fibrin sealant groups demonstrated a similar trend for peritoneal tissue generation (PTG): (i) a constant level for the first 1 day; (ii) an increase in mean volume from 1 day with control cases showing a rapid increase peaking at 14 days whereas novel fibrin sealant cases increased less severely, peaking to a plateau at 5 to 7 days; and (iii) a decrease following the peak in mean PTG volume (Graph 5.3). Novel fibrin sealant cases showed lower mean volumes throughout this study, with significant differences at 1 ($P<0.001$), 4 ($P<0.01$) and 16 hours ($P<0.001$), 3 ($P<0.001$), 7 ($P<0.05$) and 14 days ($P<0.001$) (Appendix V Table 5.1).

Control cases showed an initial increase in uterine horn tissue generation (UTG) from 1 to 4 hours followed by constant levels from 4 hours to 3 days. This in turn was followed by a rapid increase, peaking at 5 days and decreasing rapidly to 14 days, after which an increase in mean volume was seen to 42 days (Graph 5.4). Novel fibrin sealant cases showed an increase in mean volume of UTG from 1 hour to 1 day, at which peak volume occurred, with a decrease from 1 to 3 days and levels remaining constant until the end of the study. Overall, novel fibrin sealant cases demonstrated lower mean volumes of UTG compared to controls, with significant differences at 4 ($P<0.05$) hours, 1 ($P<0.05$), 5 ($P<0.001$), 7 ($P<0.001$), 14 ($P<0.05$) and 42 days ($P<0.001$) (Appendix V Table 5.1).

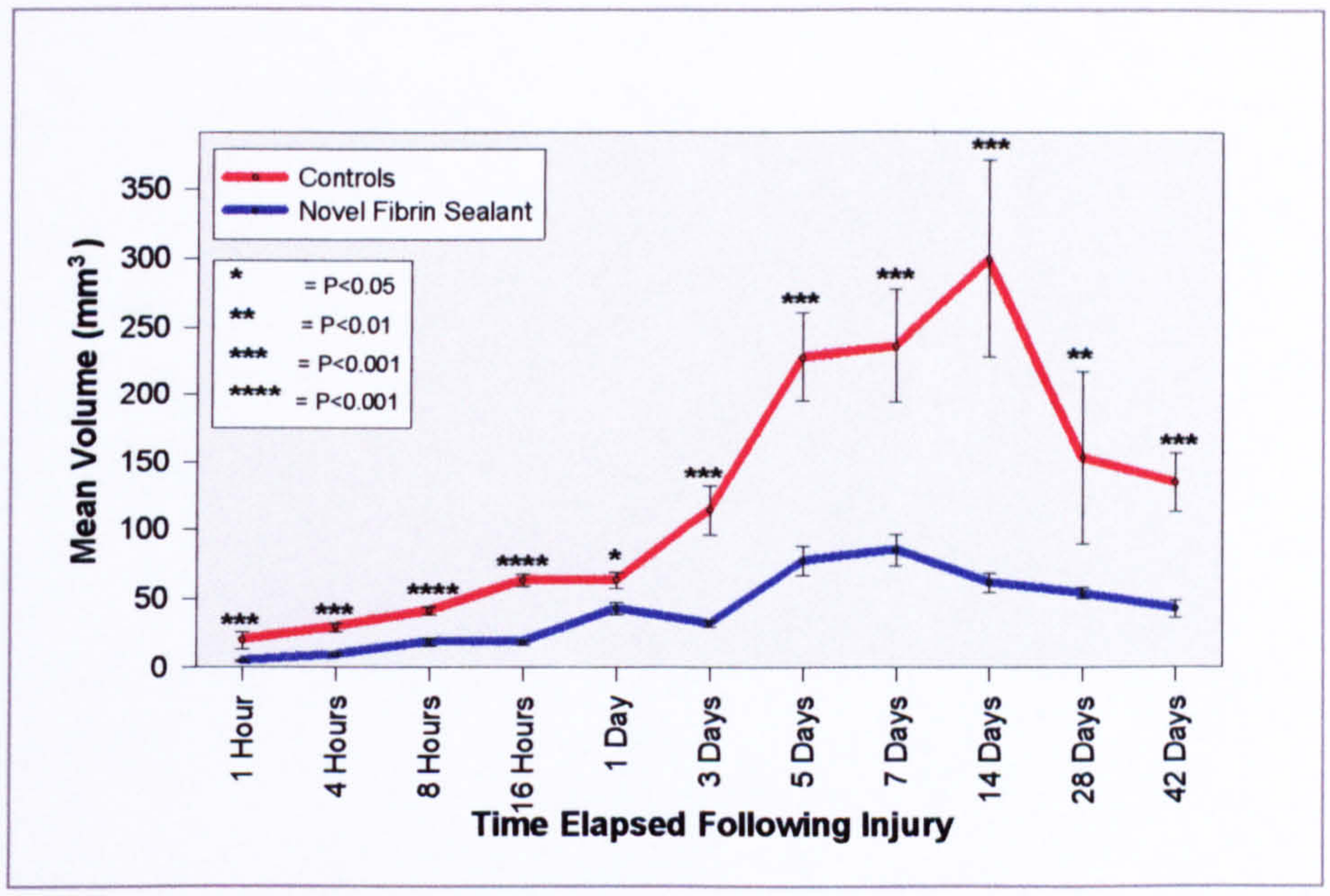
Both control and novel fibrin sealant groups showed similar trends for mean volume of total tissue generation (TTG): increasing mean volumes from 1 hour, peaking at 14 days and 5 day post injury respectively and subsequently decreasing for the remainder of the study (Graph 5.5). At all sample time points mean TTG volume in novel fibrin sealant cases was significantly less ($P<0.05$) compared to control cases (Appendix V Table 5.1).



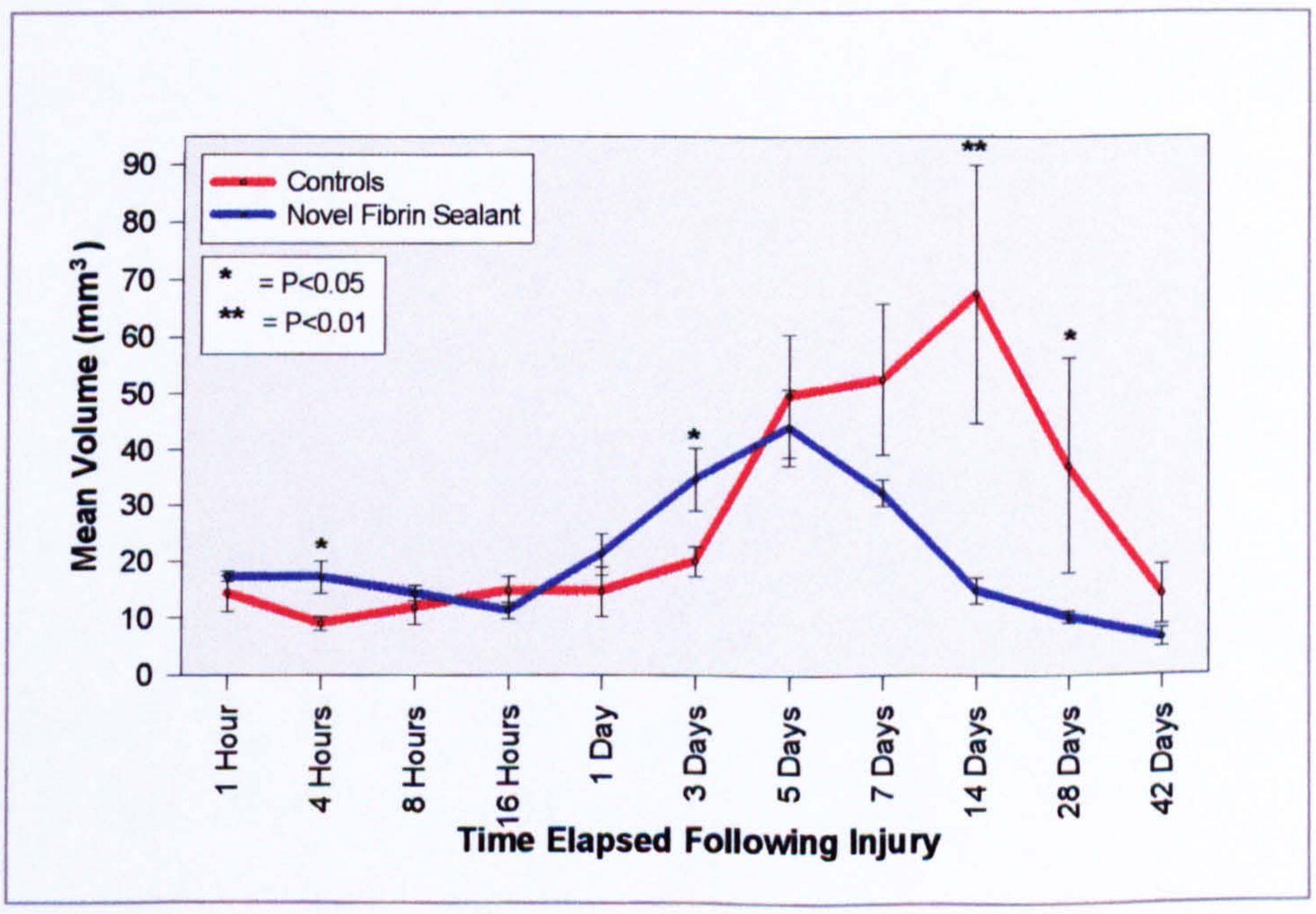
Graph 5.3: Mean PTG volumes
together with SEMs and statistical differences.



Graph 5.4: Mean UTG volumes
together with SEMs and statistical differences.



Graph 5.5: Mean TTG volumes
together with SEMs and statistical differences.

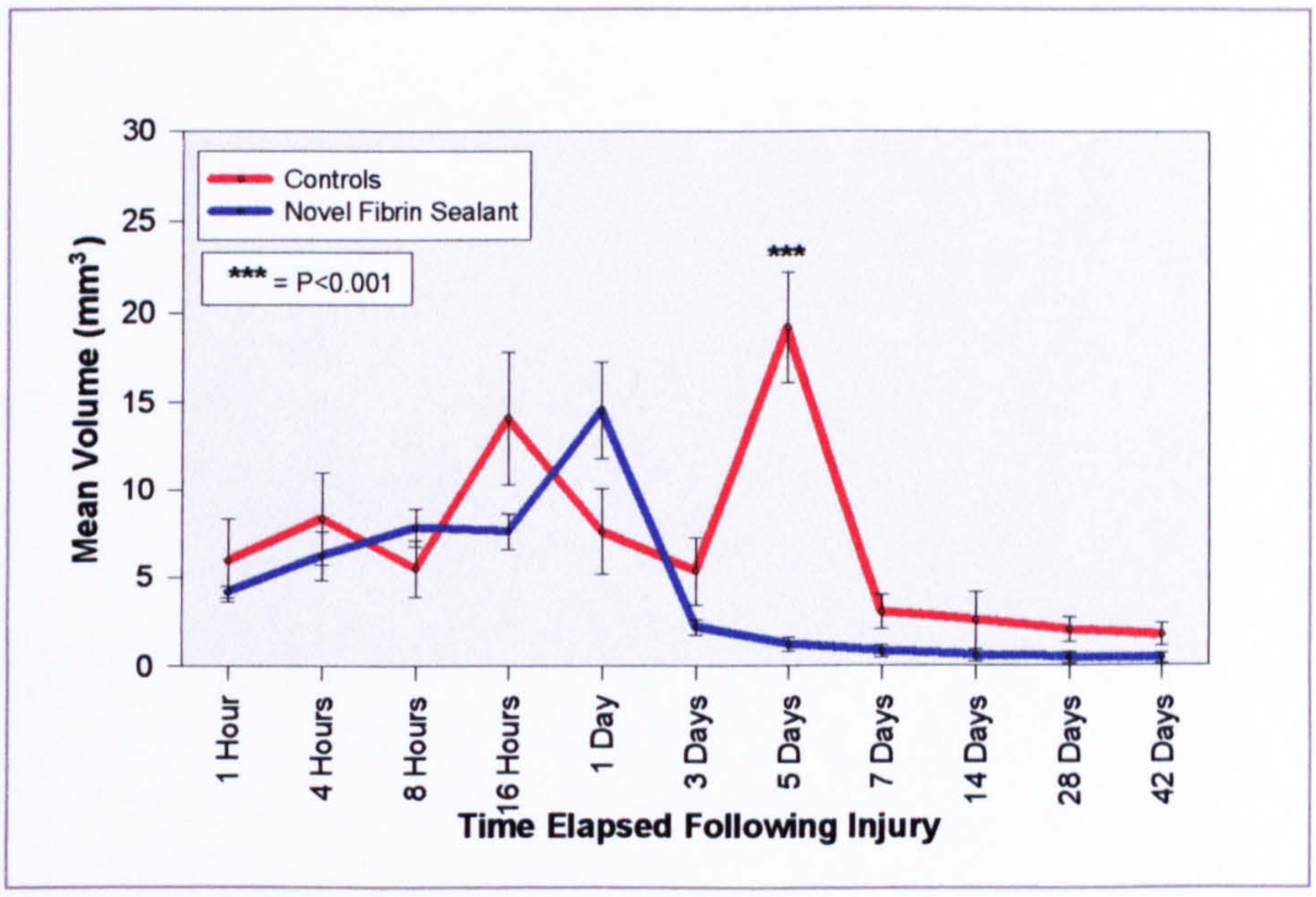


Graph 5.6: Mean peritoneal damage volumes
together with SEMs and statistical differences.

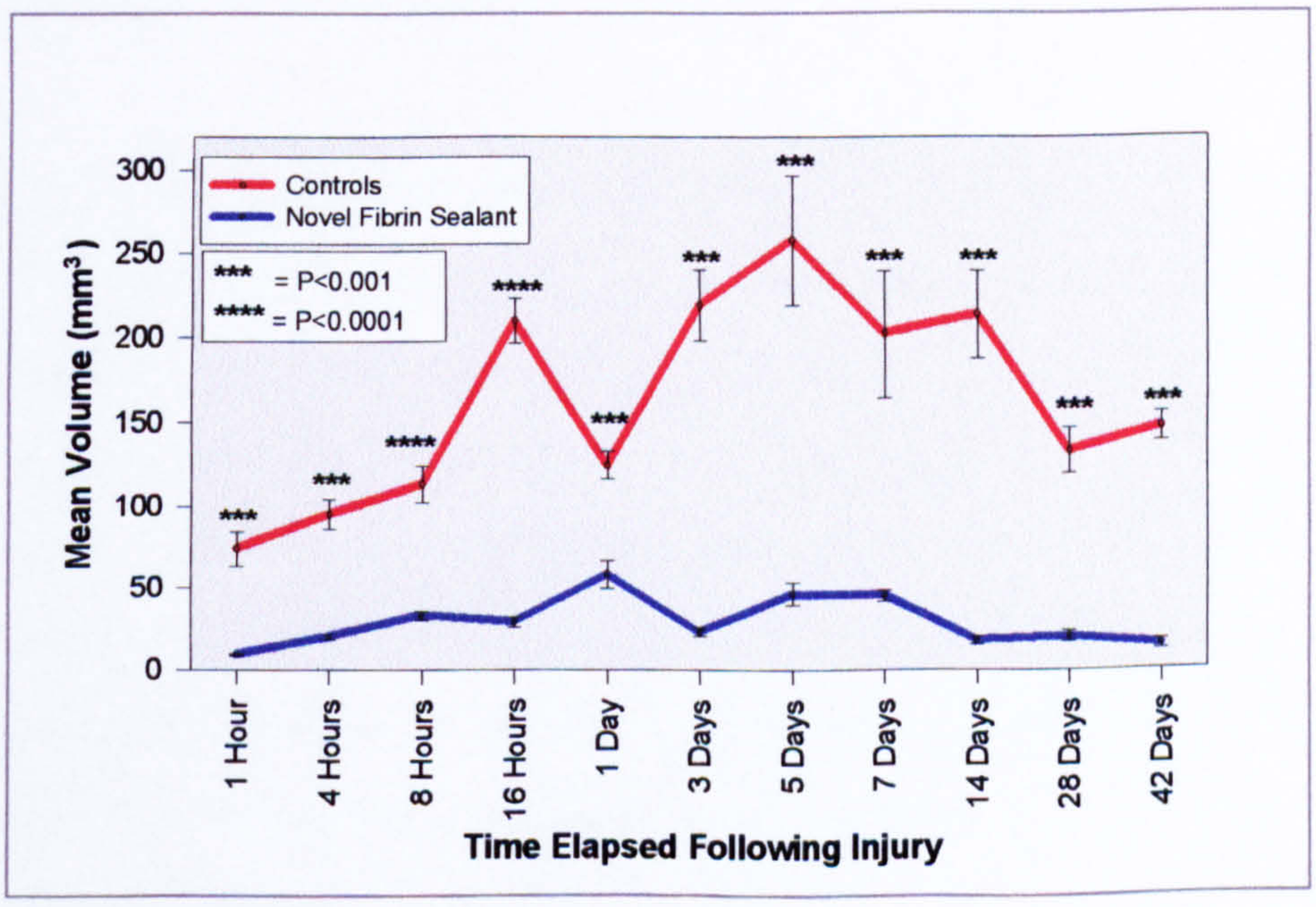
Both peritoneal and uterine horn damage demonstrated a similar pattern for both control and treated cases: constant mean volumes followed by a significant increase to peak and then subsequently decreasing. Peritoneal damage was as follows: (i) constant mean volumes from 1 hour to 3 days post injury for control cases and 16 hours for treated cases; (ii) rapid increase to peak at 14 days for control cases and 5 days for treated cases; and (iii) decrease until the end of the study, with mean volumes similar to those at 1 hour post injury (Graph 5.6). Significantly greater mean volumes ($P<0.05$) were seen in the treated group at 4 hours and 3 days whereas significantly greater volumes were demonstrated in control cases at 14 ($P<0.01$) and 28 days ($P<0.05$) post injury (Appendix V Table 5.1).

Uterine horn damage demonstrated a similar trend : (i) constant mean volumes from 1 hour to 3 days for control cases and 16 hours for treated cases; (ii) rapid increase to peak at 5 days for control cases and 1 day for treated cases; and (iii) a rapid decrease to 7 days and 3 days respectively, with a gradual decrease for the remainder of the study (Graph 5.7). From 3 days onwards treated cases showed a trend to have lower mean volumes of uterine horn damage compared to controls, being significant ($P<0.001$) at 5 days following injury (Appendix V Table 5.1).

A similar trend was seen for both control and novel fibrin sealant groups with regard to peritoneal reaction (Graph 5.8). Both groups demonstrated an increase in mean volume from the first sample time point of 1 hour, with peaks seen at 16 hours and 5 days post injury for controls and 1 and 5 to 7 days for novel fibrin sealant cases. Throughout the whole of this study, significantly lower ($P<0.001$) mean volumes of peritoneal reaction were seen for novel fibrin sealant cases compared to controls.



Graph 5.7: Mean uterine horn damage volumes
together with SEMs and statistical differences.

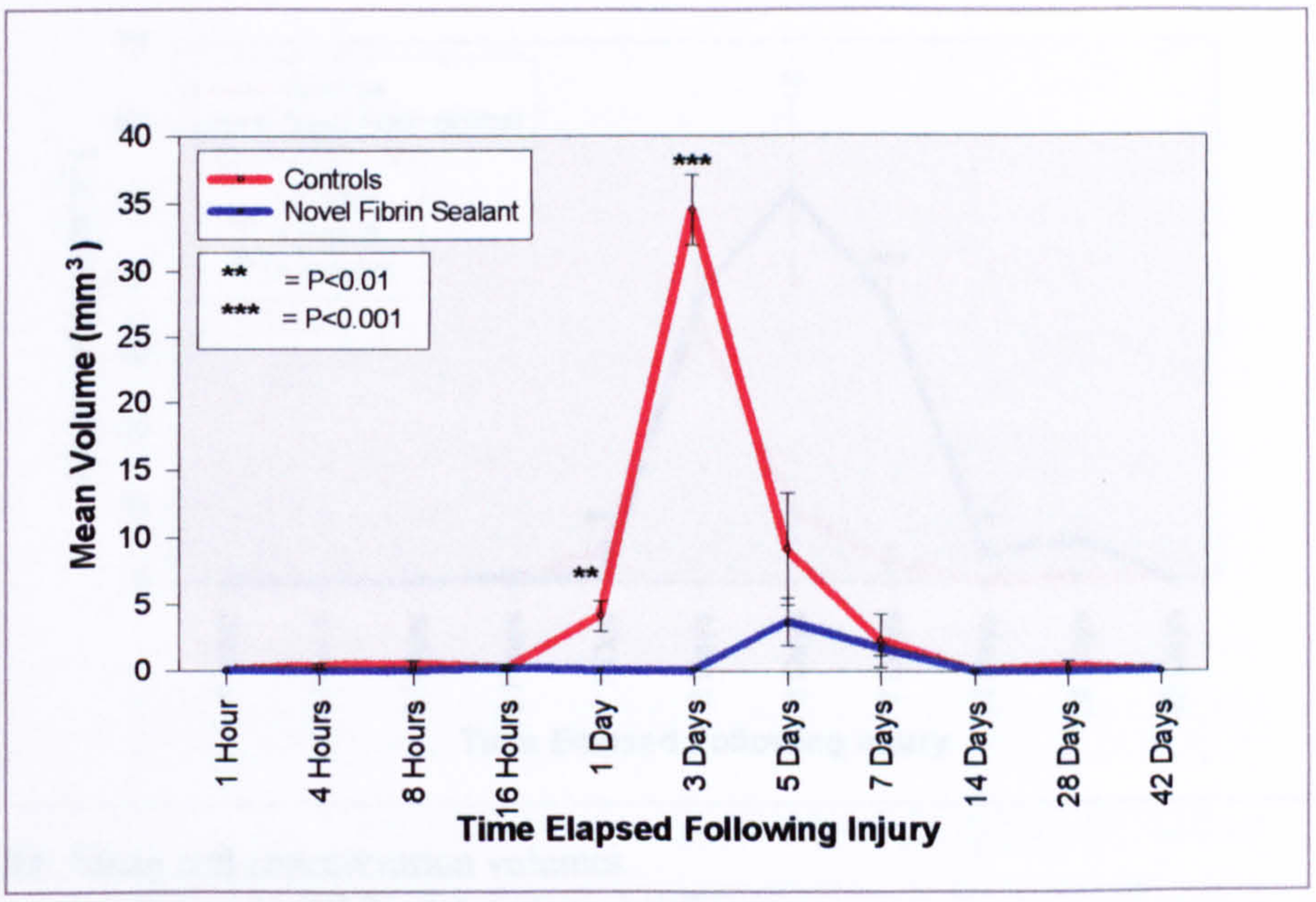


Graph 5.8: Mean peritoneal reaction volumes
together with SEMs and statistical differences.

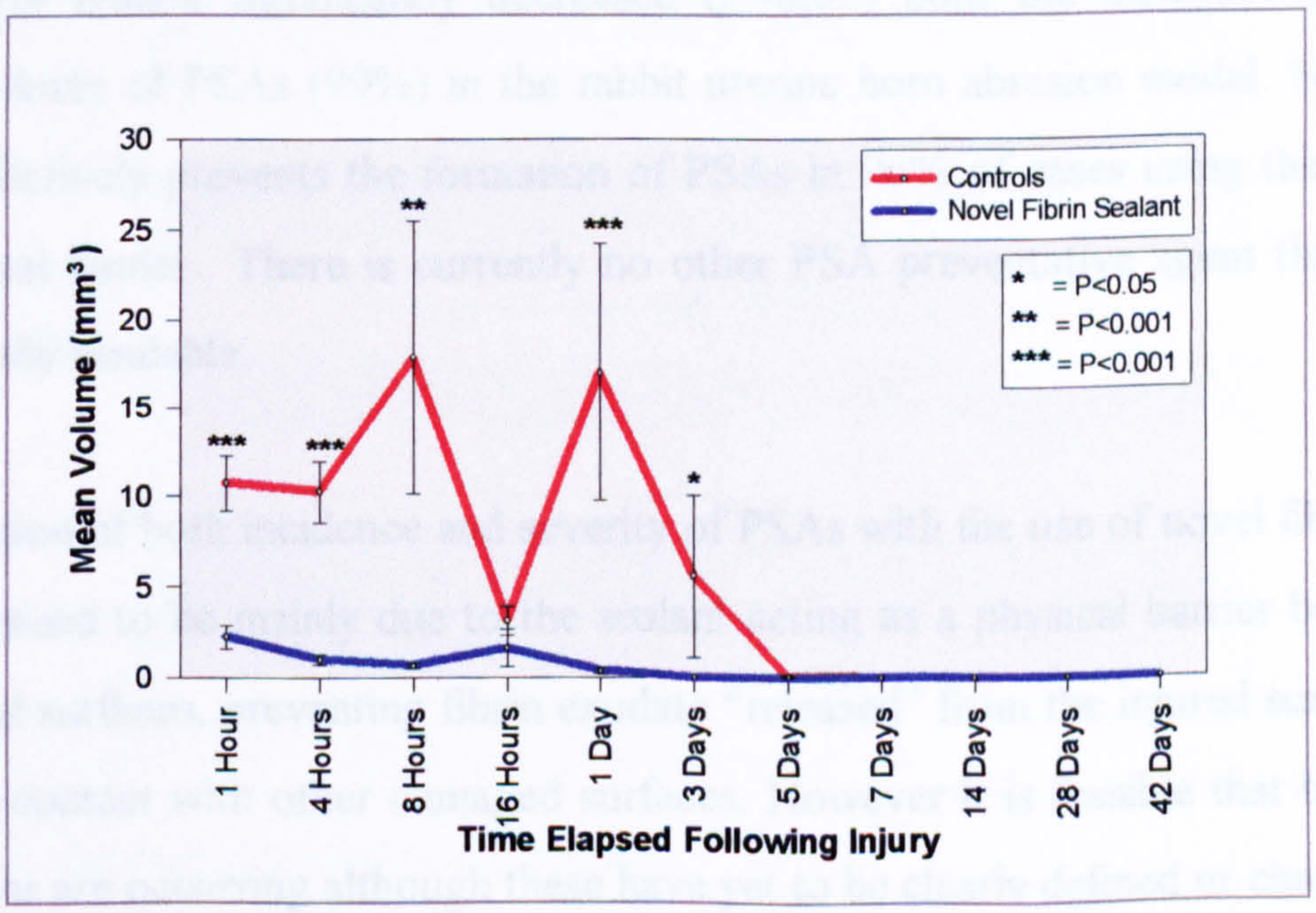
Peritoneal cell concentration also showed very similar patterns for control and novel fibrin sealant groups: initially constant/absent levels, followed by an increase to a peak and then decreasing until levels are again absent (Graph 5.9). Controls showed an increase between 16 hours and 3 days, with the peak at 3 days followed by a decrease and levels seen up to 14 days post injury. In contrast novel fibrin sealant cases showed an increase from 3 to 5 days where mean volume peaked, and a decrease up to 14 days, with levels absent thereafter. Mean volume of peritoneal cell concentration for novel fibrin sealant cases were again less than control cases, with significant differences ($P<0.01$) at 1 and 3 days following injury (Appendix V Table 5.1).

Overall novel fibrin sealant cases demonstrated a lower extent of uterine horn haemorrhage compared to controls, with significant differences ($P<0.05$) at 1, 4, 8 hours and 1 and 3 days (Graph 5.10; Appendix V Table 5.1). Control cases showed major peaks in mean volume at 8 hours and 1 day whereas novel fibrin sealant cases demonstrated minor peaks at 1 and 16 hours.

Mean total cell concentrations, peritoneal cell concentration for control cases and peritoneal cell concentration and cell concentration associated with fibrin sealant for novel fibrin sealant cases, showed similar trends throughout this study (Graph 5.11). Controls showed minimal levels, increasing rapidly from 1 day to peak at 3 days following injury, with levels decreasing rapidly thereafter. Novel fibrin sealant cases also increased rapidly from 1 day but peaked at 5 days, with levels decreasing gradually to 7 days and then rapidly thereafter. Significantly greater mean volume ($P<0.01$) for control cases compared to treated cases was seen at 1 day post injury, whereas significantly greater volumes were seen for treated cases at 5 ($P<0.01$), 7 ($P<0.001$) and 14 days ($P<0.05$) (Appendix V Table 5.1).



Graph 5.9: Mean peritoneal cell concentration volumes together with SEMs and statistical differences.



Graph 5.10: Mean uterine horn haemorrhage volumes together with SEMs and statistical differences.

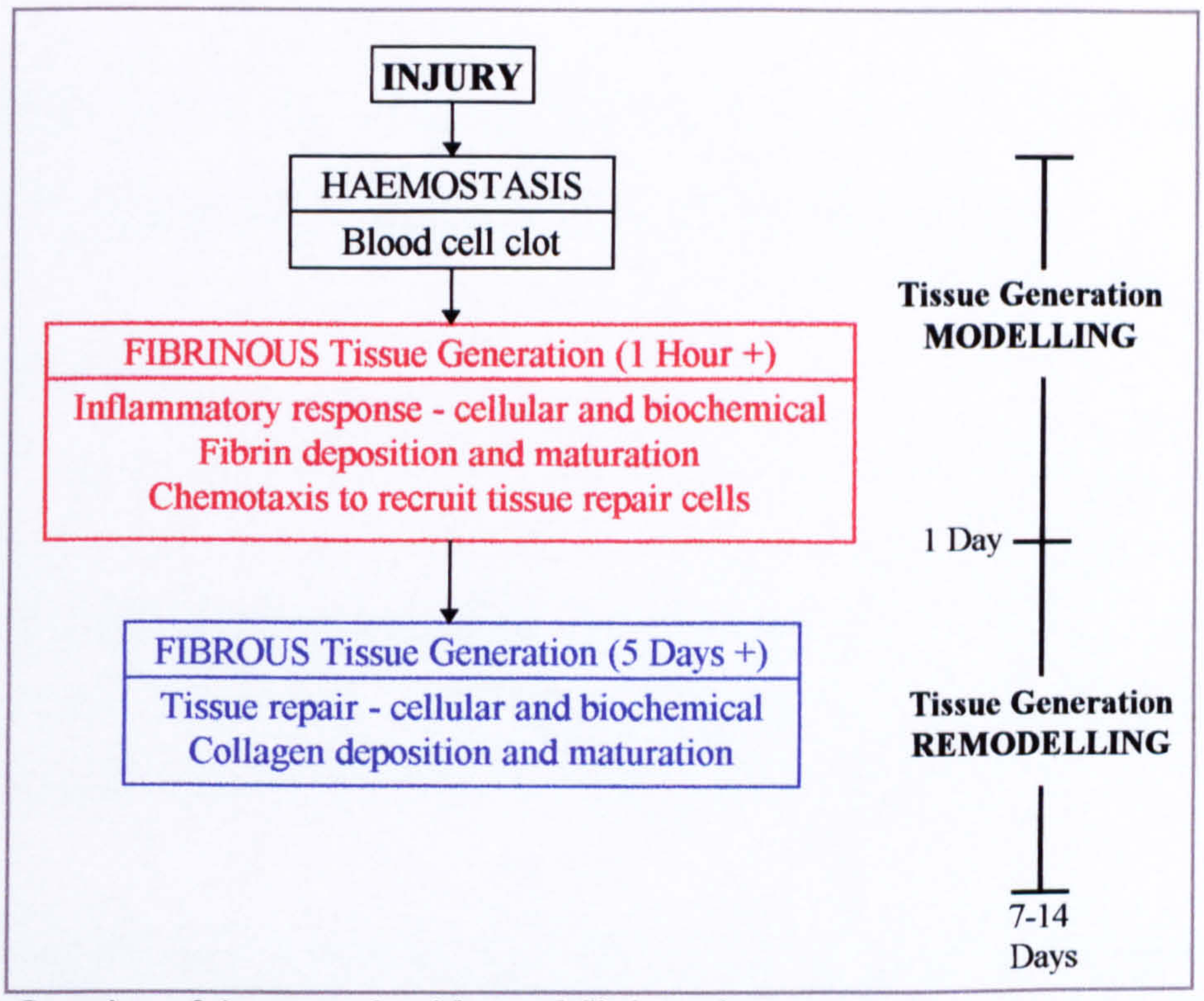


Figure 4.16: Overview of tissue repair with novel fibrin sealant treatment.

In vivo volume of novel fibrin sealant remained relatively constant for the first 5 days following application, after which there was a rapid loss, due to either phagocytosis and/or fibrinolysis. Therefore novel fibrin sealant was present during tissue generation modelling, fibrin deposition, and during the initial stages of tissue generation remodelling, collagen deposition. Novel fibrin sealant presence at injury site(s) during these stages is highly important and probably the major reason behind its PSA prevention success.

A cellular response to human novel fibrin sealant in this rabbit experimental model was evident from 3 to 7 days post injury, with maximum response seen at 5 days. This response primarily consisted of macrophages and eosinophilic cells. It is hypothesised that this is an immune mediated response to the presence of foreign protein within the peritoneum, with phagocytic cells attracted to phagocytose this foreign material. Such a response should be absent when novel fibrin sealant is used as intended, that is, as a truly autologous sealant.

pattern of wound healing. It does however exert the effect of decreasing the volume of physiological changes and the time taken for such changes to occur (Figure 5.1).

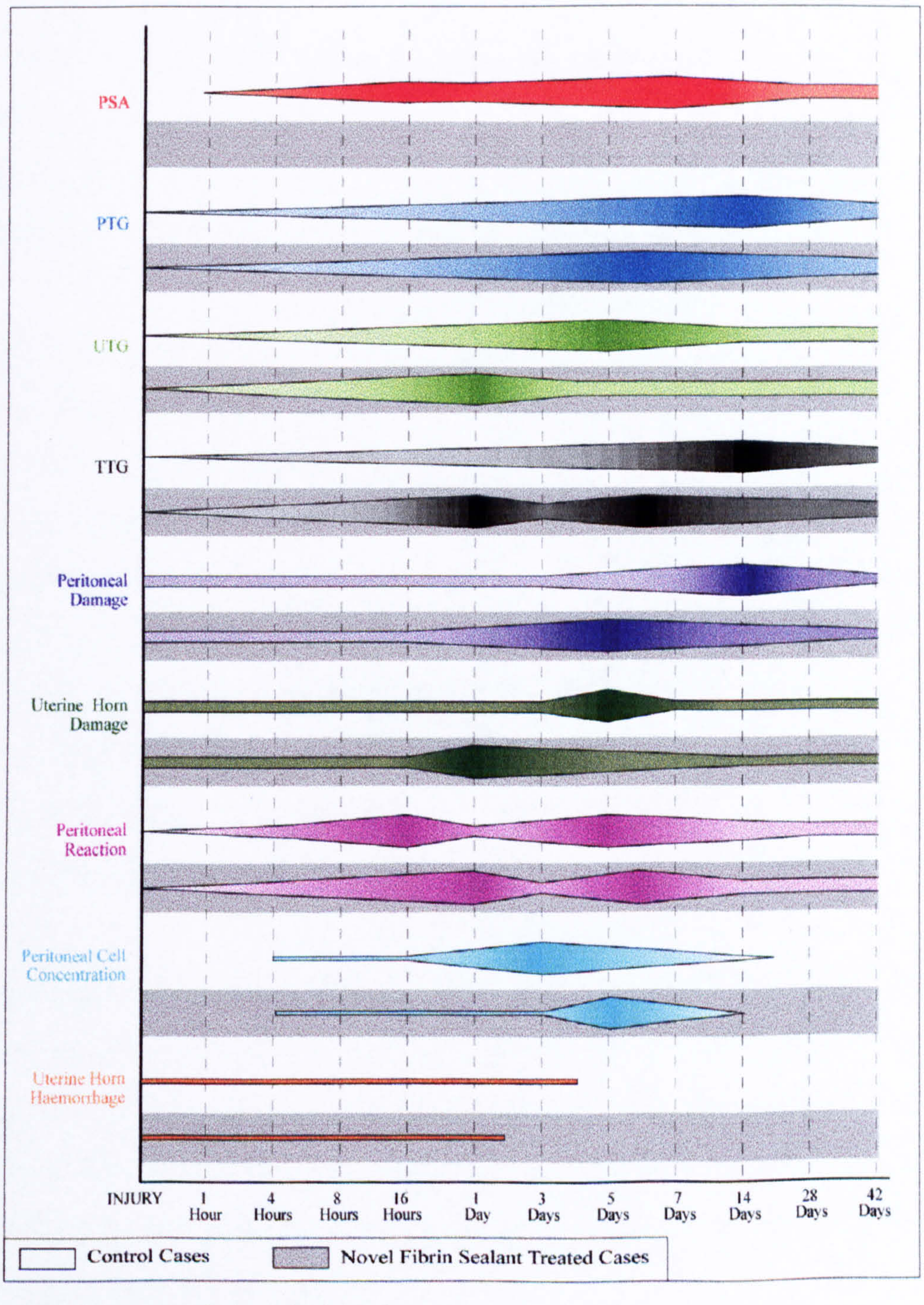


Figure 5.1: Summary of physiological changes.

We know that immediately following injury blood loss will be stopped by haemostasis, with a platelet plug and fibrin clot formed. Platelets present will secrete chemical mediators which attract cells and the inflammatory process is initiated. Tissue surrounding and underlying the abrasion injury will have sustained a level of damage and will subsequently undergo necrosis. In order for proper wound healing to occur this necrotic tissue is removed by phagocytes, with volume of damage increasing and increased volume of tissue generation to replace this removed tissue. Once completed, damage and tissue generation volumes decrease as injured tissue is repaired.

From the data obtained during this study we know that the volume of damage sustained from the abrasion process was the same for control and treated cases. In treated cases subsequent decreases in volumes are due to alterations in the response to injury, that is, a decreased inflammatory reaction, decreased necrosis of surrounding and underlying tissue and in turn tissue generation. This decreased necessity for wound healing response after treatment with novel fibrin sealant in turn decreases the time required for tissue repair to occur. Reasons for limitation of wound healing time include: (i) novel fibrin sealant acting as a physical barrier preventing cellular and biochemical interactions between peritoneal and uterine horn and their interactions with the peritoneal cavity; and/or (ii) biochemical modulation of wound healing by novel fibrin sealant.

Although significantly lower mean volumes of peritoneal reaction were seen in treated cases compared to control cases, a large cellular reaction to the presence of human blood derived novel fibrin sealant was seen. Although this does not impinge on the prevention of PSAs in this model, such concentrations of inflammatory and phagocytic cells may play additional roles which are not evident in this study. Biochemical investigations are recommended to clarify this further.

Conclusions

i. In this rabbit uterine horn abrasion model novel fibrin sealant:
    a. prevented PSAs formation in 96% of cases
    b. decreased the volume and severity of PSAs by 99%
    c. did not alter the pattern of wound healing
    d. decreased the extent of physiological changes and subsequently the time taken for those changes to occur
ii. Mechanisms of action for PSA prevention and alterations of wound healing by novel fibrin sealant include:
    a. sealant acting as a physical barrier, preventing fibrin exudate from joining injured surfaces and, in turn, preventing interactions between surfaces and peritoneal contents
    b. biochemical modulations by the sealant.

5.3 Volumetric Analysis of Fibrin and Collagen

In order to further define the mechanisms in which novel fibrin sealant prevents the formation of PSAs, fibrin and collagen presence within injury tissue will be directly compared between control and novel fibrin sealant treated cases.

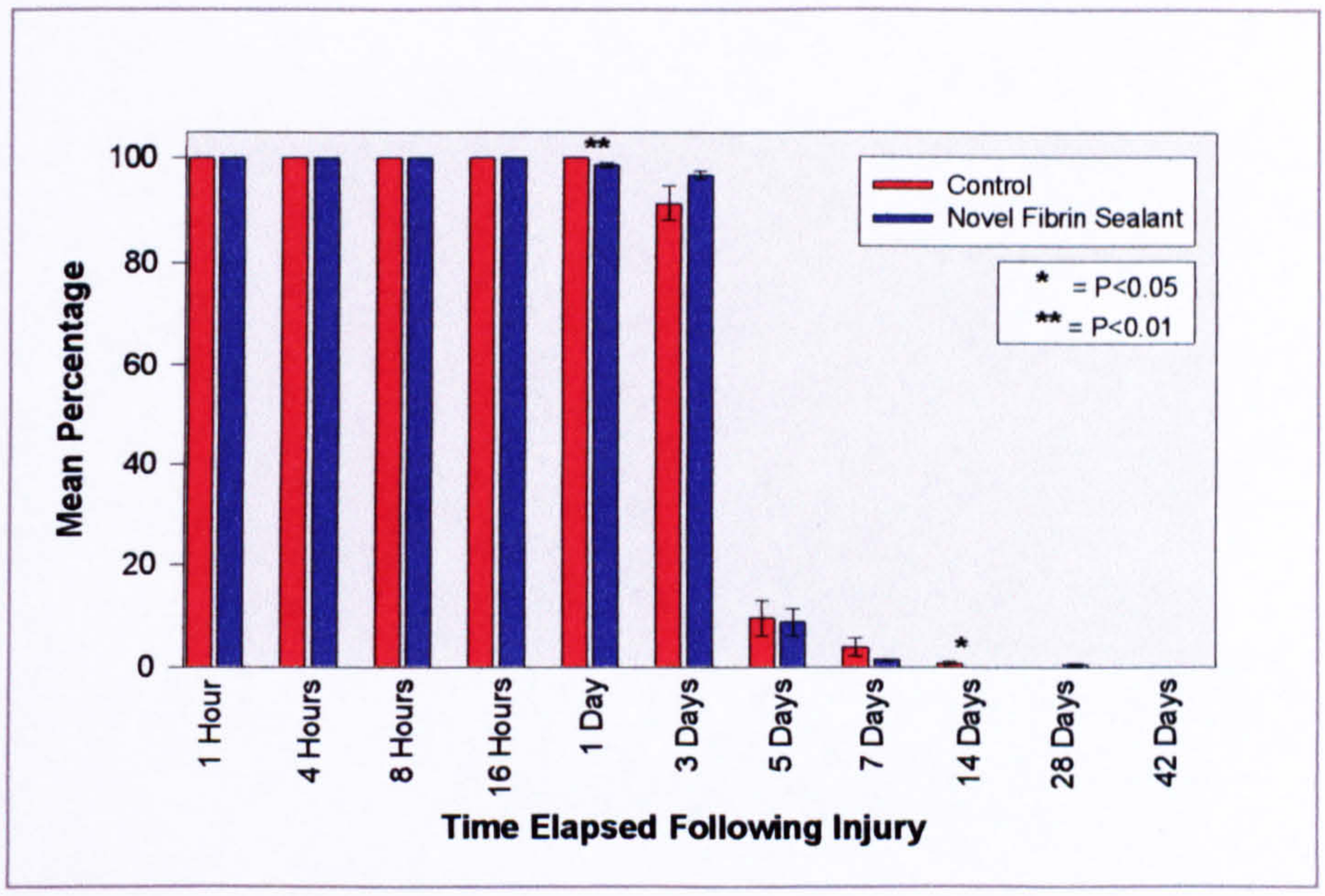
5.3.1 Results

Mean percentages of fibrin and collagen for control and novel fibrin sealant treated cases demonstrated very similar patterns, with fibrin predominant during the first 3 days and collagen predominant from 5 days onwards (Graphs 5.12 and 5.13). During the first 16 hours post injury fibrin only was present in total injury tissue generated after which mean percentage decreased slightly to 3 days and then rapidly from 3 to 5 days, to be absent from 28 days onwards. In contrast collagen percentages were *vice versa*: first present in minimal percentages at 1 days, rapidly increased from 3 to 5 days, with collagen only present from 28 days and thereon. Significant differences between control and treated cases were seen at 1 and 14 days following injury with treated cases demonstrating significantly lower percentages ($P<0.01$ and $P<0.05$ respectively) of fibrin and therefore

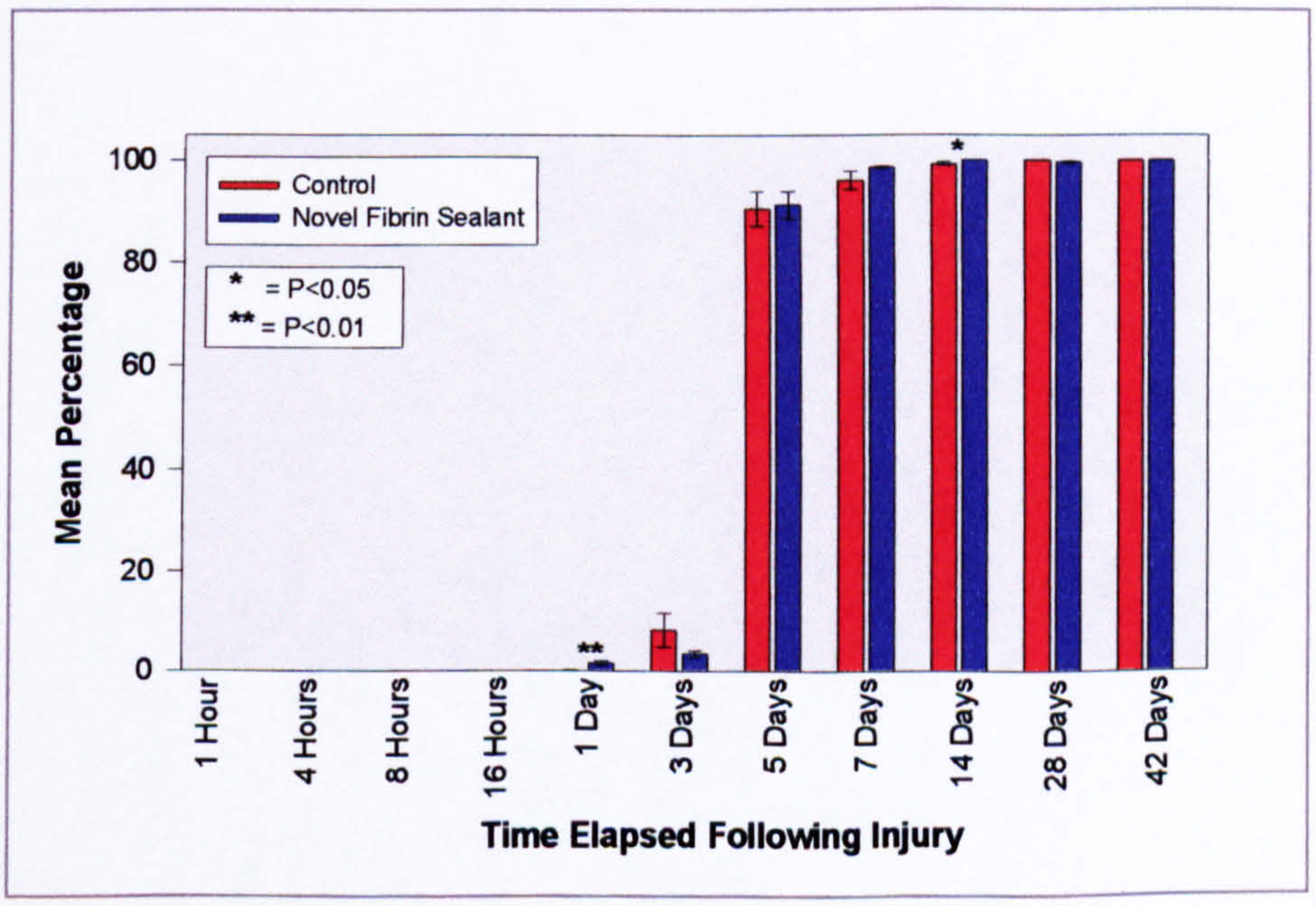
significantly greater percentages of collagen compared to control cases (Appendix V Table 5.2).

Mean volumes of fibrin for control and treated cases demonstrated similar trends throughout this study: (i) steadily increasing mean volumes from 1 hour to 1 day for control cases and 16 hours for treated cases; (ii) rapid increase to a peak in mean volume at 3 and 1 day respectively; (iii) rapid decrease to 5 days for both groups; and (iv) steadily decreasing mean volumes until absent at 28 days post injury (Graph 5.14). Overall, mean volume of fibrin was greater in the control group compared to treated group, with significantly greater volumes at 4 ($P<0.05$), 8 ($P<0.01$), 16 hours ($P<0.01$), 3 ($P<0.001$) and 14 days ($P<0.05$) post injury (Appendix V Table 5.2).

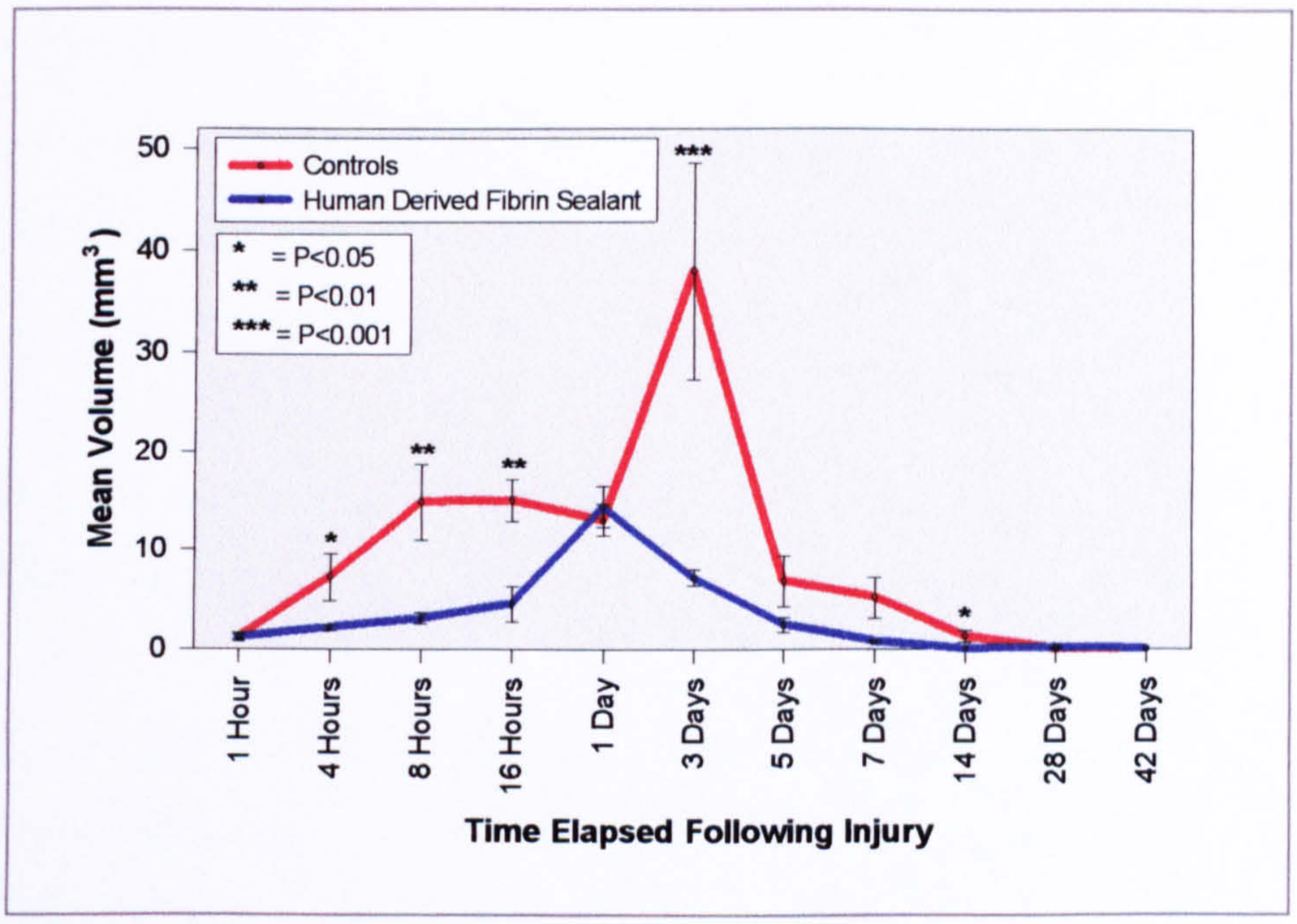
Control and treated groups also demonstrated a similar pattern for collagen mean volumes: (i) absent for the first 16 hours; (ii) minimal volumes at 1 and 3 days; (iii) rapid increase to peak at 14 days for control group and 7 days for treated group; (iv) decrease in mean volume to 42 days for control group and 14 days for treated group, with level remaining similar until 42 days for the later group (Graph 5.15). Throughout this study mean volume of collagen was greater in the control group compared to treated, with significantly greater mean volumes at 1 ($P<0.01$), 3 ($P<0.05$), 5 ($P<0.05$), 7 ($P<0.01$), 14 ($P<0.001$) and 42 days ($P<0.01$) (Appendix V Table 5.2).



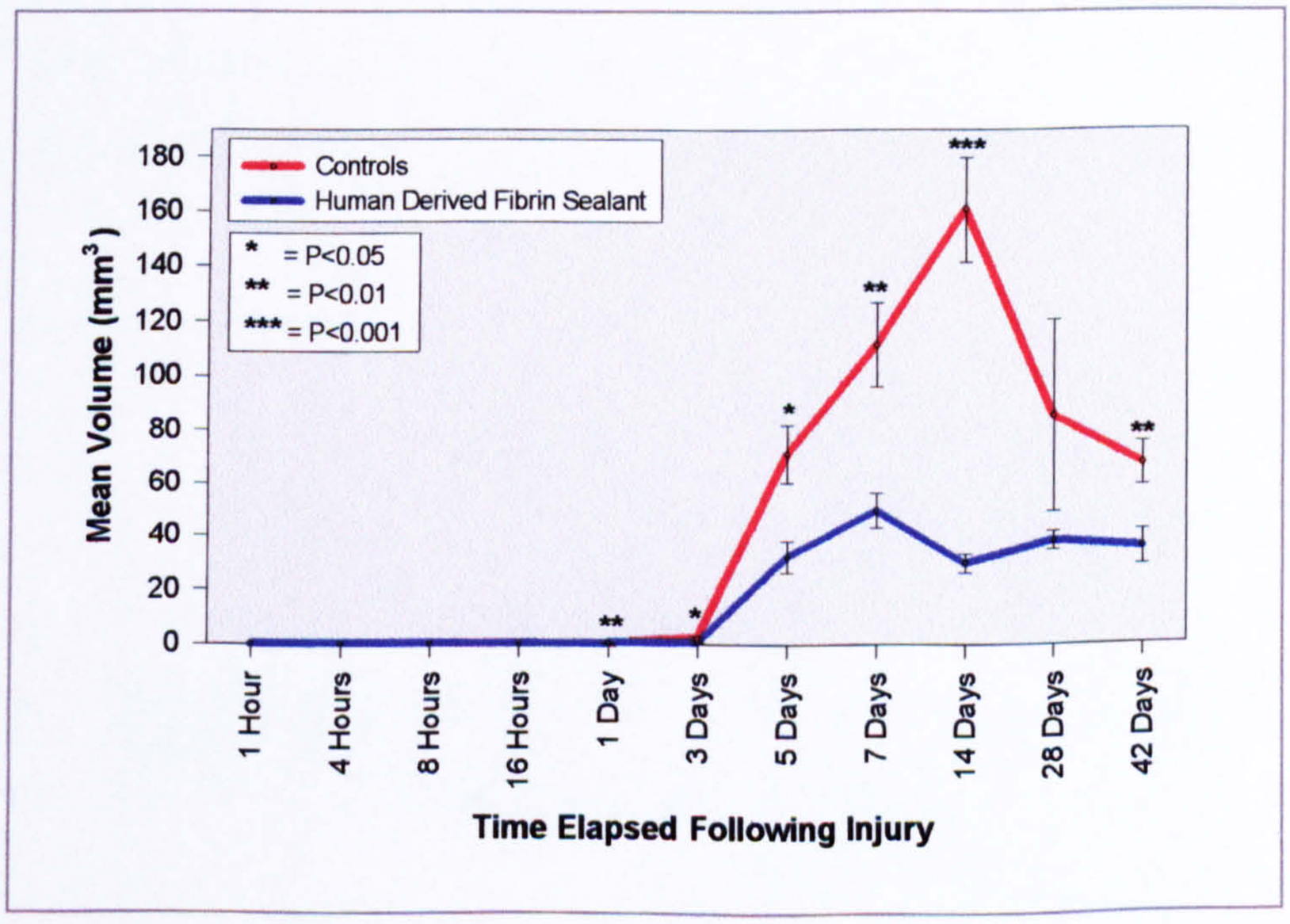
Graph 5.12: Mean fibrin percentages
together with SEMs and statistical differences.



Graph 5.13: Mean collagen percentages
together with SEMs and statistical differences.



Graph 5.14: Mean fibrin volumes
together with SEMs and statistical differences.



Graph 5.15: Mean collagen volumes
together with SEMs and statistical differences.

5.3.2 Discussion

Both control and novel fibrin sealant treated cases demonstrated the same patterns for fibrin and collagen presence and predominances in total tissue generated in response to injury: fibrin predominant for the first 3 days with collagen predominant from 5 days onwards. Therefore tissue generated in response to injury was fibrinous for the first 3 days and fibrous from 5 days onwards whether injury sites were left untreated or treated with novel fibrin sealant.

Although novel fibrin sealant did not exert any major effect on the pattern of structural development of injury tissue, minor alterations were seen (Figure 5.2). From 1 to 14 days following injury greater mean percentages of collagen were seen in treated cases compared to controls, being significant ($P<0.05$) at 1 and 14 days. Thus treatment with novel fibrin sealant accelerated the presence of collagen in injury tissue due to increasing fibroblast migration and/or fibroblast synthesis of collagen and promoted an accelerated loss of fibrin. Hypotheses for this effect include: (i) novel fibrin sealant acting as a physical barrier preventing interactions between injured surfaces and between injured surfaces and peritoneal contents; and/or (ii) biochemical modulation of wound healing by the sealant.

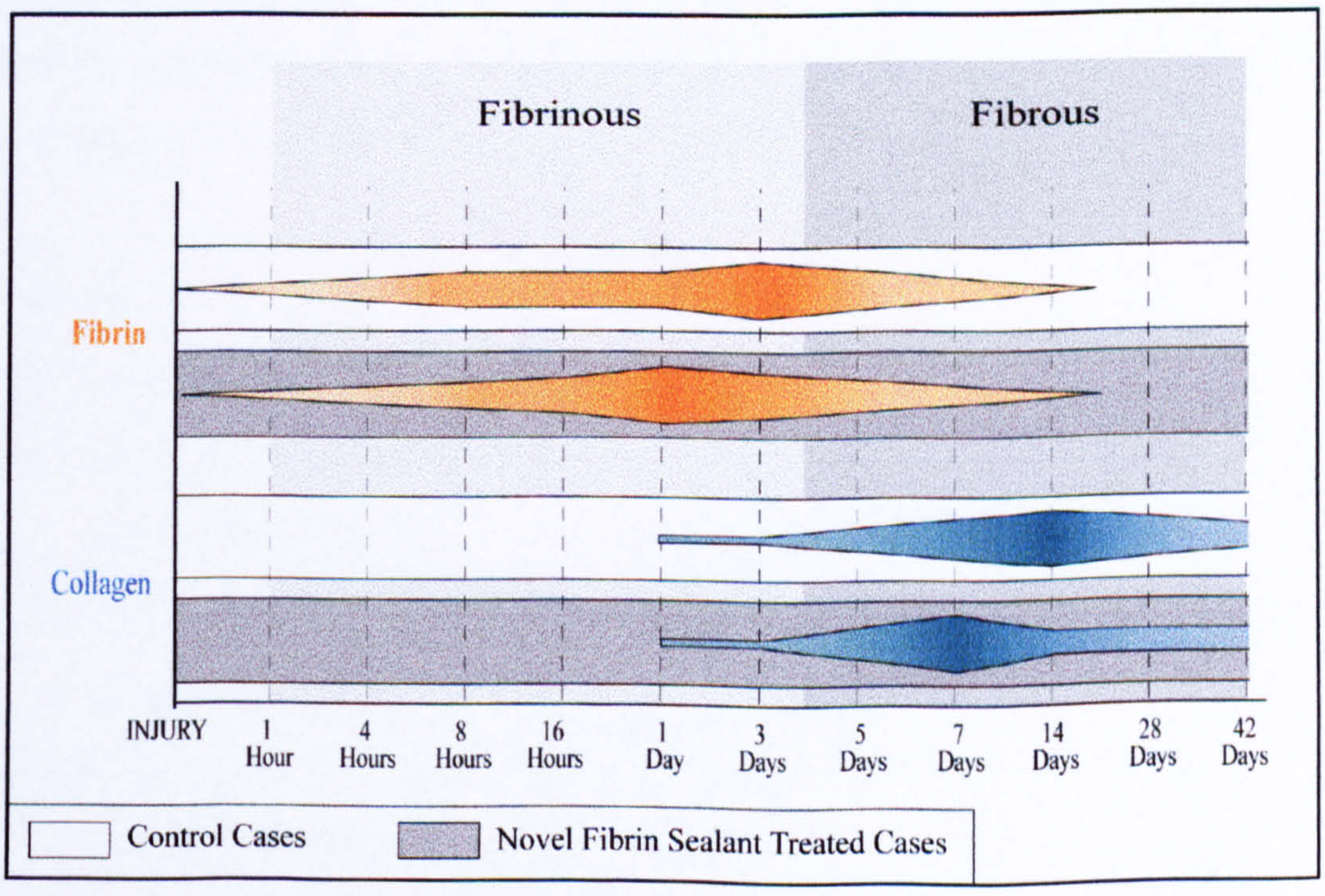


Figure 5.2: Summary of structural changes.

The lower mean volumes of fibrin and collagen in treated cases compared to controls was due to the lower mean volume of tissue generated in response to injury by treated cases (refer to Section 5.2).

Patterns for fibrin mean volumes were similar for control and treated cases: fibrin laid down to prevent blood loss and protect tissues from the peritoneal environment occurring from injury to 3 days for controls and 1 day for treated cases, with decreasing volumes thereafter. Treatment with novel fibrin sealant decreased the time required for maximum fibrin presence and subsequent loss. Cellular and biochemical activity are the stimulators for fibrin deposition following haemostasis and it is likely that these were altered by the presence of novel fibrin sealant. Whether this alteration was due to direct biochemical modulation by novel fibrin sealant or due to isolation of injured tissues is as yet undetermined.

Control and treated cases also demonstrated a similar pattern for collagen presence: absent during the first 16 hours, increasing mean volumes to peak at 14 days for controls and 7 day for treated cases, with subsequent decreasing volumes finally reaching a plateau in treated cases only. Hence novel fibrin sealant accelerated both collagen synthesis and maturation. This increased rate of tissue repair is hypothesised to be due to novel fibrin sealant decreasing the inflammatory response and necrotic damage following injury, leading to tissues being available for repair at an earlier time.

Conclusions

i. Treatment with novel fibrin sealant:
    a. does not alter the pattern of structural protein presence and predominance's within injury tissues
    b. accelerated maximum fibrin presence and fibrin loss
    c. accelerated collagen synthesis and maturation
ii. Mechanisms of action of novel fibrin sealant responsible for these changes could be:
    a. the sealant acting as a physical barrier preventing interactions between injured surfaces and between injured surfaces and peritoneal contents; and/or

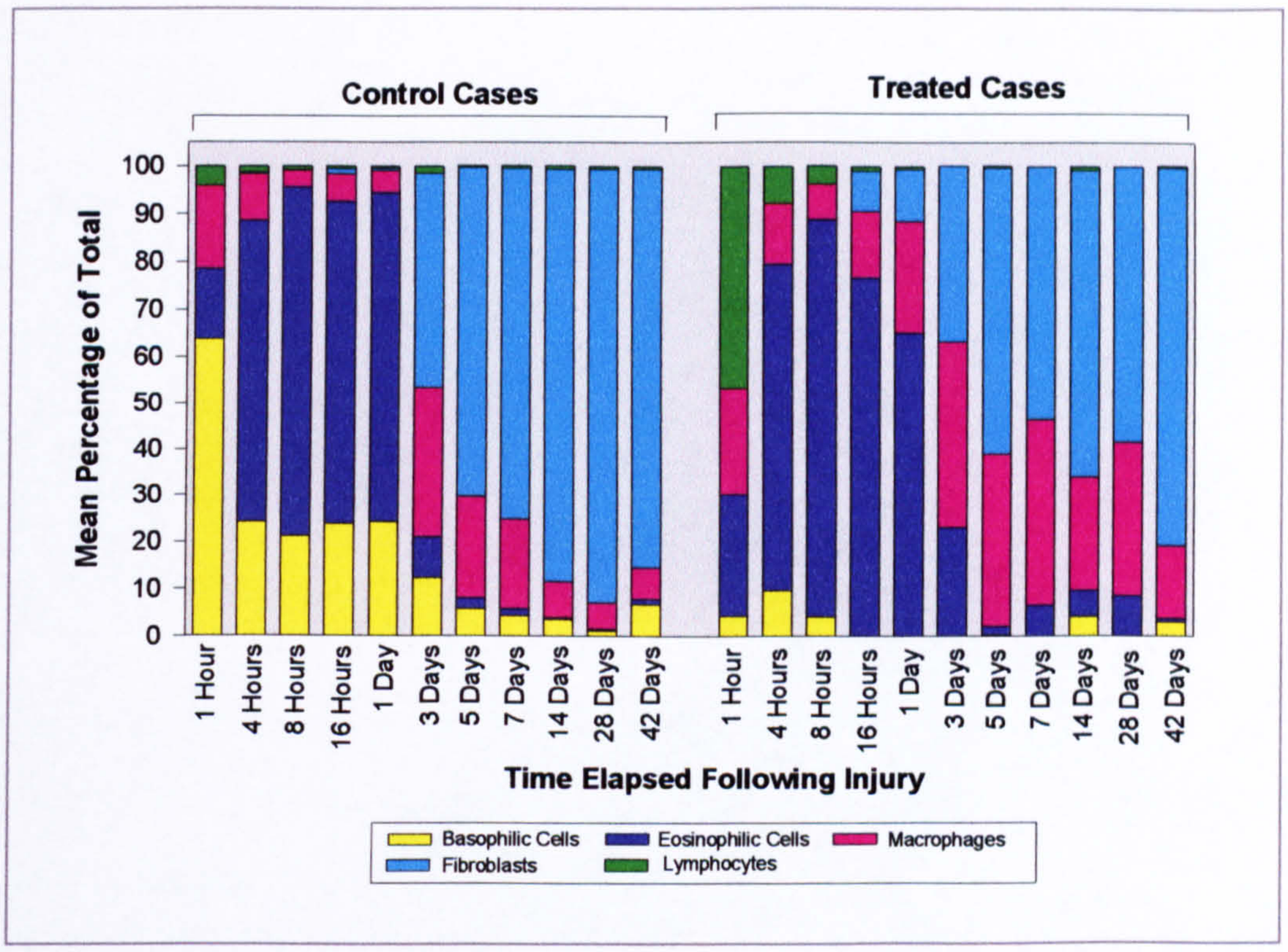
b. biochemical modulation of wound healing by the sealant.

5.4 Number Analysis of Cellular Components

Cellular involvement is essential for wound healing, PSA development or PSA prevention. In order to gain further understanding into the actions of novel fibrin sealant direct comparisons between control and treated cases is required with regard to cellular presence.

5.4.1 Results

Cell predominances for number densities followed similar trends for control and treated cases (Graph 5.16). For the first day following injury inflammatory cells (eosinophilic cells, basophilic cells and macrophages) were the predominant cell type, with tissue repair cells (fibroblasts and macrophages) being predominant from 3 day onwards. Minor differences between groups were present: control cases demonstrated greater percentages of basophilic cells throughout the study whereas treated cases demonstrated greater percentages of lymphocytes from 1 to 8 hours, fibroblasts at 16 hours and 1 day and macrophages throughout the study.



Graph 5.16: Cell predominances for number densities.

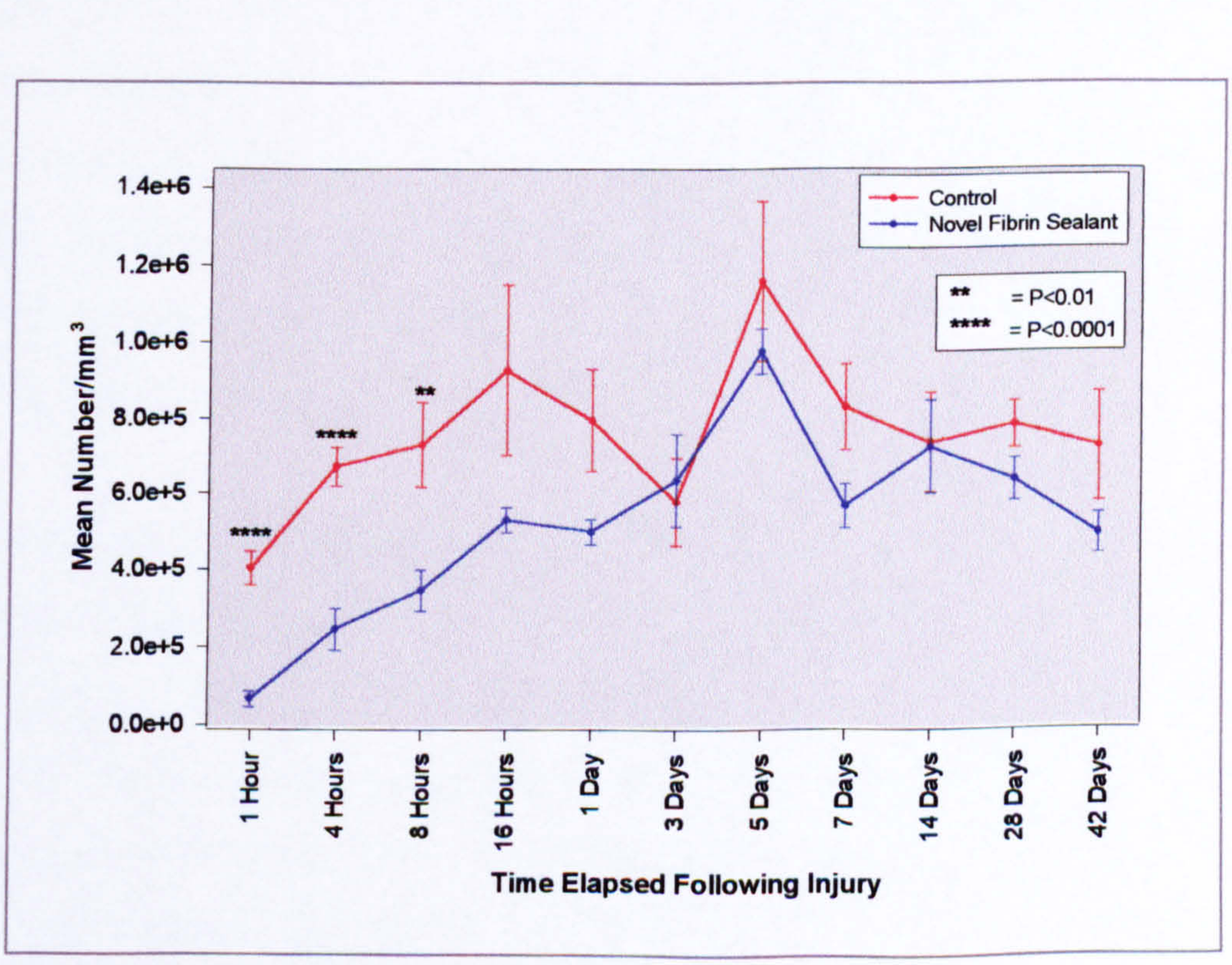
Since similar trends for both number densities and total were demonstrated in PSAs, PTG and UTG for control and treated cases these injury tissue zones were grouped together as total tissue generation. In addition basophilic and eosinophilic cells were grouped together as granulocytes.

5.4.1.1 Number Densities

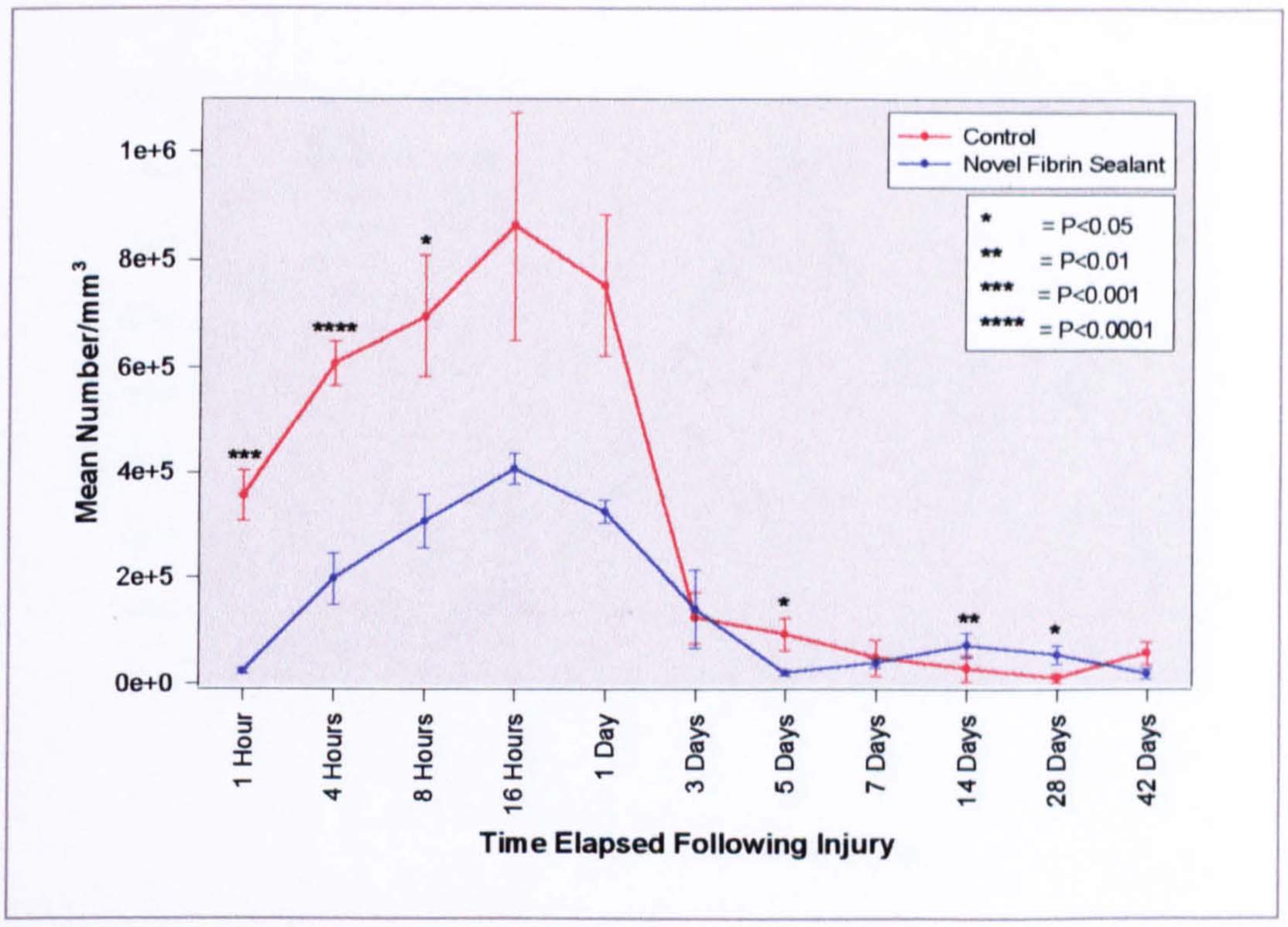
Both control and treated cases demonstrated similar trends with regard to total cell densities within TTG throughout this study (Graph 5.17). From 1 hours post injury mean densities increased to peak at 16 hours, with a decrease from 1 to 3 days for control cases whereas an increase was seen for treated cases. Both groups demonstrated increasing mean densities from 3 day, with a second peak at 5 days, with densities showing a trend to decrease until 42 days. Throughout the whole study control case mean densities were greater then treated cases, followed by significantly greater densities at 1 ($P<0.0001$), 4 ($P<0.0001$), and 16 hours ($P<0.01$) post injury (Appendix V Table 5.3).

Mean densities of granulocytes (basophilic and eosinophilic cells) followed a similar trend to total cells. Densities increased from 1 hour to peak at 16 hours, with a sharp decline to 3 days and constant densities thereafter (Graph 5.18). From 1 to 5 days control densities were greater then treated, being significant at 1 ($P<0.001$), 4 ($P<0.0001$) and 8 hours ($P<0.05$) and 5 days ($P<0.05$). Although both control and treated case densities were relatively constant from 5 days onwards, treated cases showed densities slightly above control cases with significantly greater values at 14 ($P<0.01$) and 28 days ($P<0.05$) post injury (Appendix V Table 5.3).

The overall pattern for macrophage densities was the same for control and treated cases. From 1 hour to 1 day for controls and to 8 hours for treated cases constant densities were seen, with a rapid increase thereafter to peak at 5 days with densities decreasing for the remainder of the study (Graph 5.19). From 16 hours onwards macrophage densities in treated cases were greater than those in control cases with this being significant at 1 ($P<0.05$), 14 ($P<0.01$) and 28 days ($P<0.0001$) (Appendix V Table 5.3).



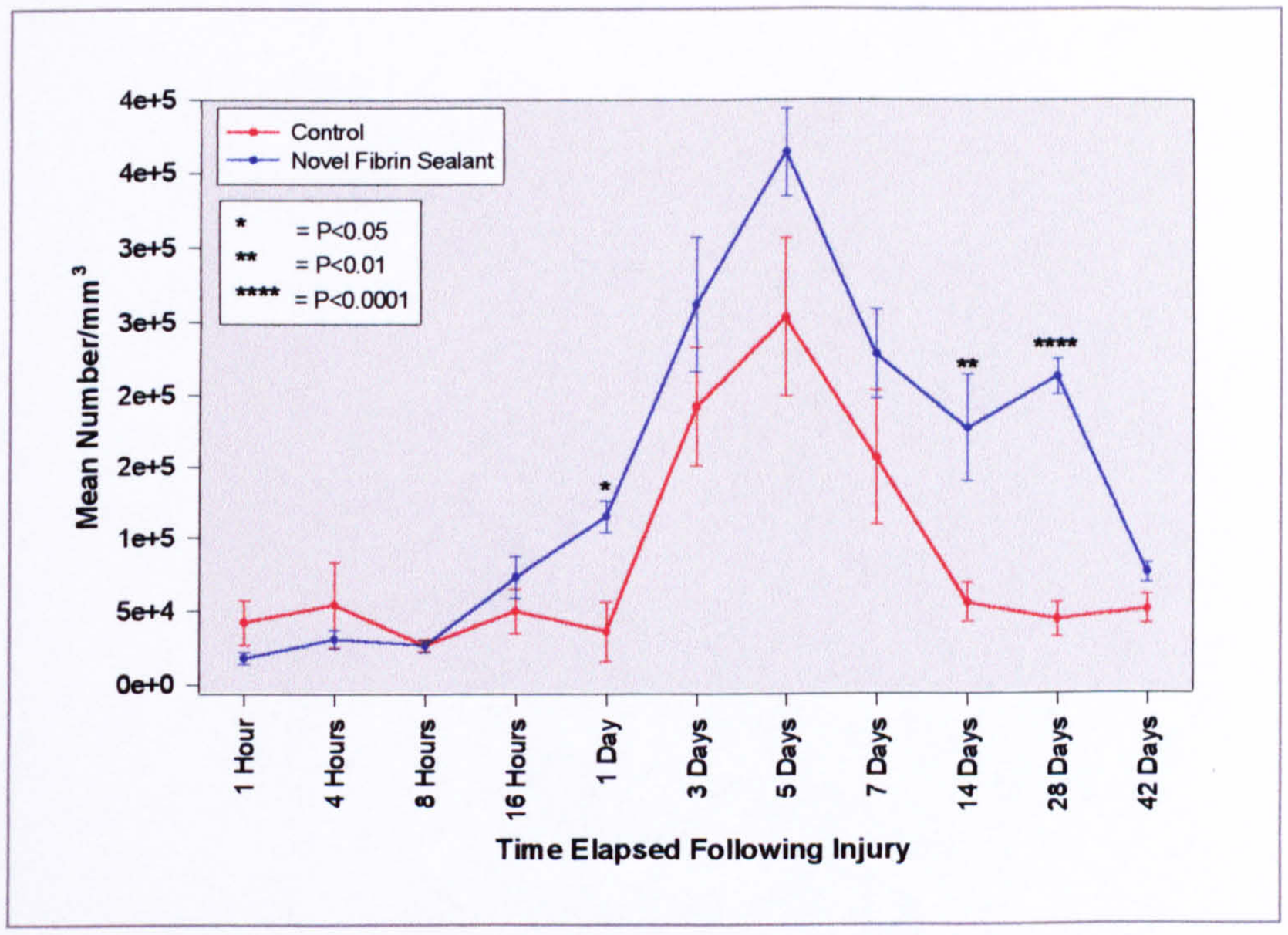
Graph 5.17: Mean total cell densities throughout TTG development together with SEMs and statistical differences.



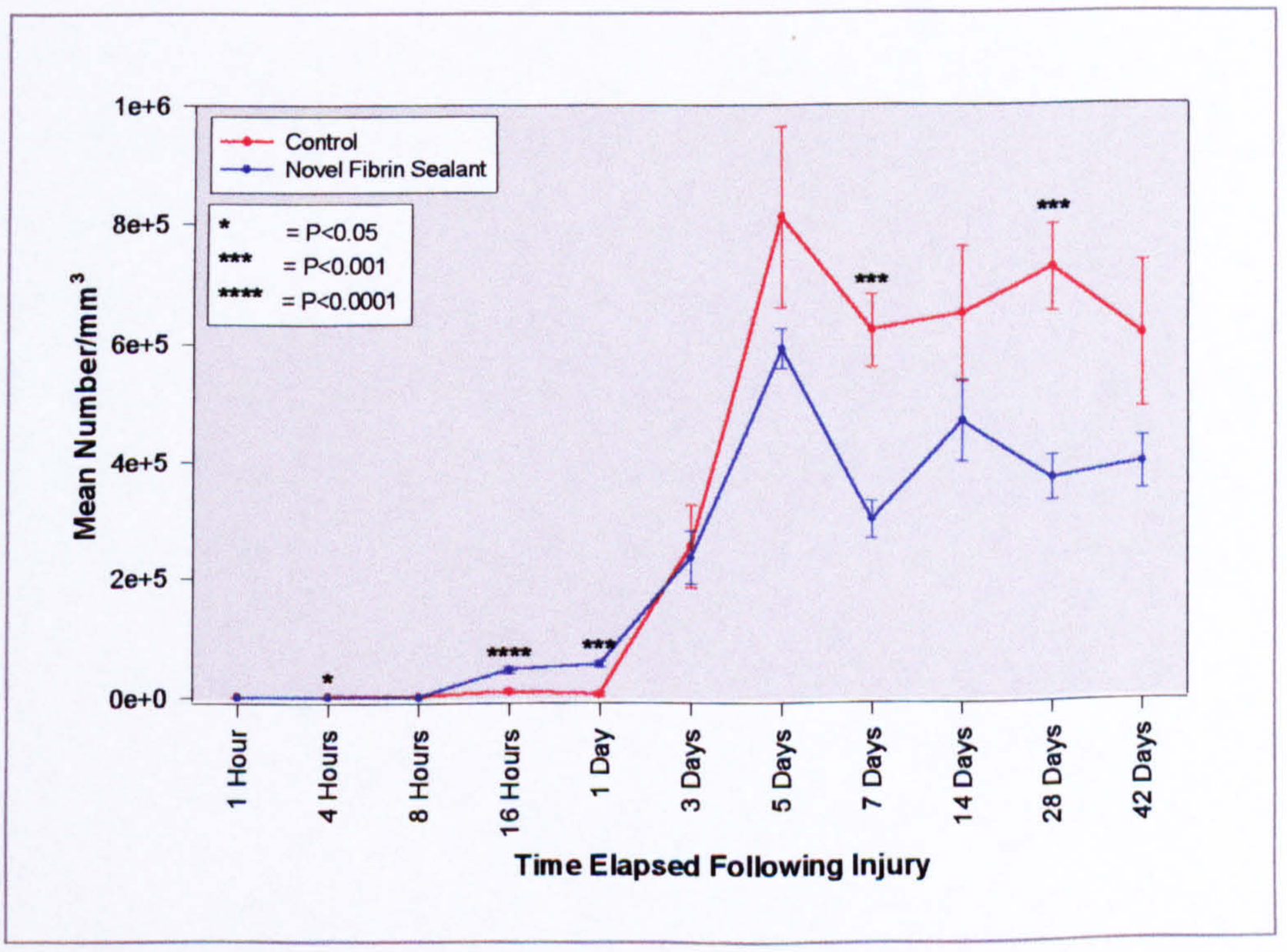
Graph 5.18: Mean granulocyte densities throughout TTG development together with SEMs and statistical differences.

Fibroblast densities were constantly low from 1 hour to 1 day for control and to 8 hours for treated cases (Graph 5.20). In treated cases density increased from 8 hours to 1 day with a rapid increase from 1 to 5 days in both groups. Following the peak at 5 days there was a decrease to 7 days with densities remaining stable thereafter. Significantly greater fibroblast densities were seen in treated cases at 16 hours ($P<0.0001$) and 1 day ($P<0.001$) whereas greater densities were seen in control cases at 4 hours ($P<0.05$) and 7 and 14 days ($P<0.01$) (Appendix V Table 5.3).

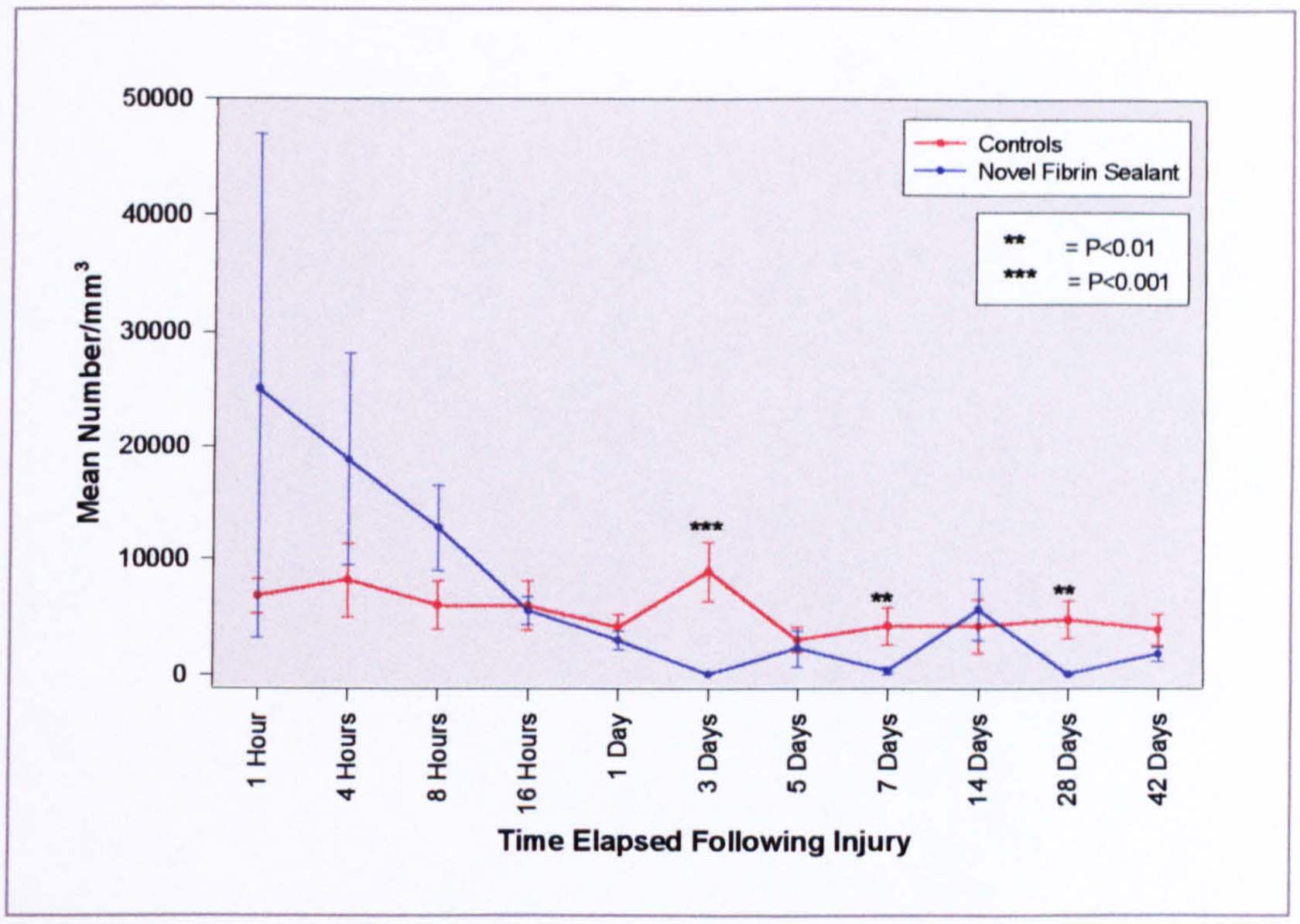
Lymphocyte density in control cases demonstrated little change throughout the course of this study, with only a minor peak at 3 days post injury (Graph 5.21). However treated cases peaked at 1 hour with a decrease to 3 days and only mild fluctuations from then on. From 1 to 8 hours lymphocyte densities in treated cases exceeded those in control cases, with the situation reversed from 1 to 42 days. Significantly greater densities were seen with control cases at 3 ($P<0.001$), 7 ($P<0.01$) and 14 days ($P<0.01$) following injury (Appendix V Table 5.3).



Graph 5.19: Mean macrophage densities throughout TTG development together with SEMs and statistical differences.



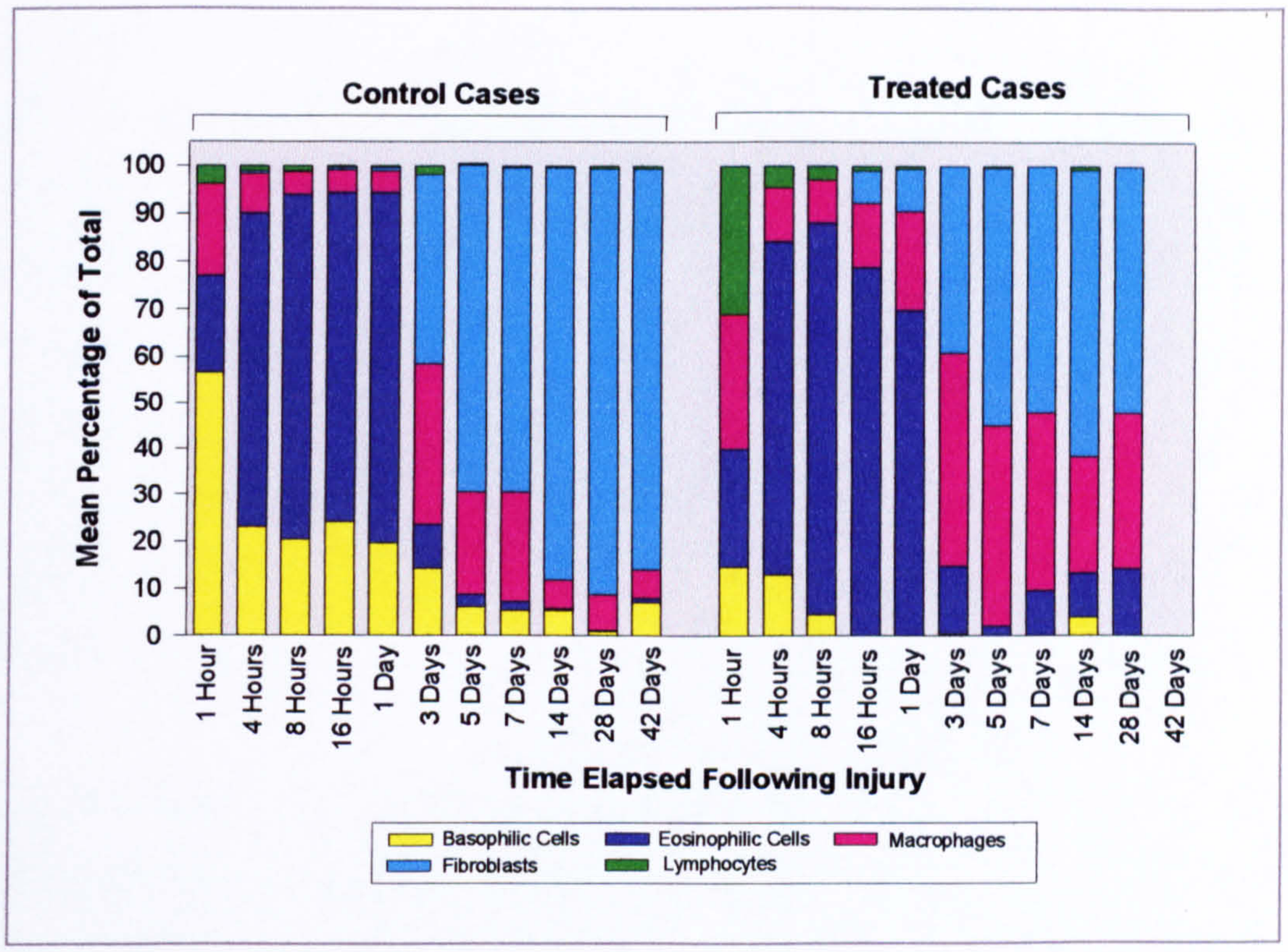
Graph 5.20: Mean fibroblast densities throughout TTG development together with SEMs and statistical differences.



Graph 5.21: Mean lymphocyte densities throughout TTG development together with SEMs and statistical differences.

5.4.1.2 Number Totals

Cell predominances for number totals followed a similar trend to those seen for number densities. Inflammatory cells (eosinophilic cells, basophilic cells and macrophages) were predominant for the first day whereas tissue repair cells (fibroblasts and macrophages) were predominant from 3 days onwards for both control and treated groups (Graph 5.22). Differences between groups included: greater percentages of basophilic cells for controls throughout this study; greater percentages of lymphocytes from 1 to 8 hours, fibroblasts at 16 hours and 1 day and macrophages throughout the study in the treated group.



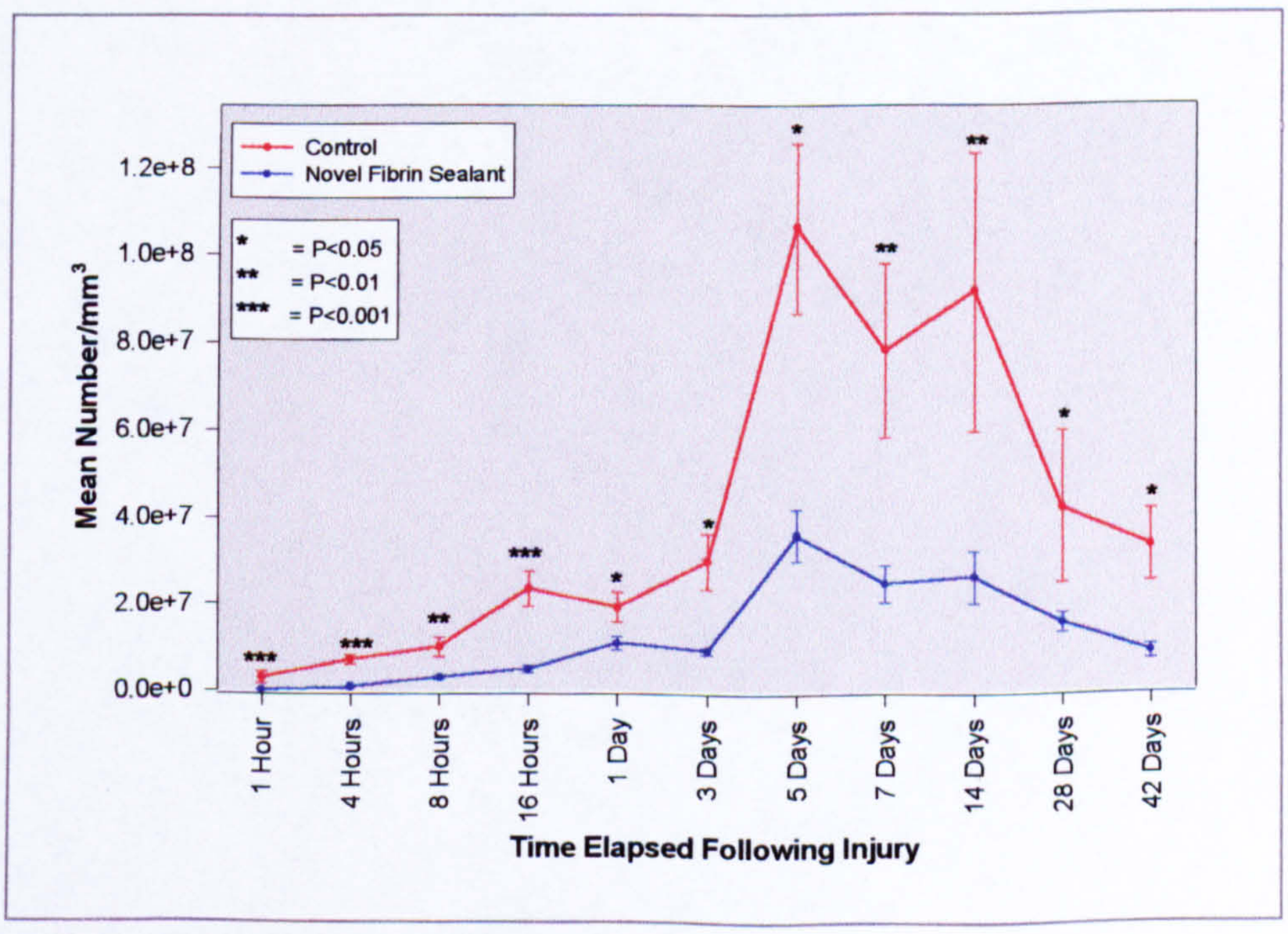
Graph 5.22: Cell predominances for number totals.

Control and treated cases both demonstrated a similar pattern for total cell numbers (cells analysed) throughout this study (Graph 5.23). From 1 hour to 3 days there was a steady increase in mean numbers, with control cases only showing a minor peak at 16 hours. A rapid increase was seen from 3 days with numbers peaking at 5 day post injury. Following this peak numbers decreased but with a minor peak at 14 days in both groups. At all time points investigated control cases demonstrated significantly greater ($P<0.05$) total cell numbers compared to novel fibrin sealant treated cases (Appendix V Table 5.4).

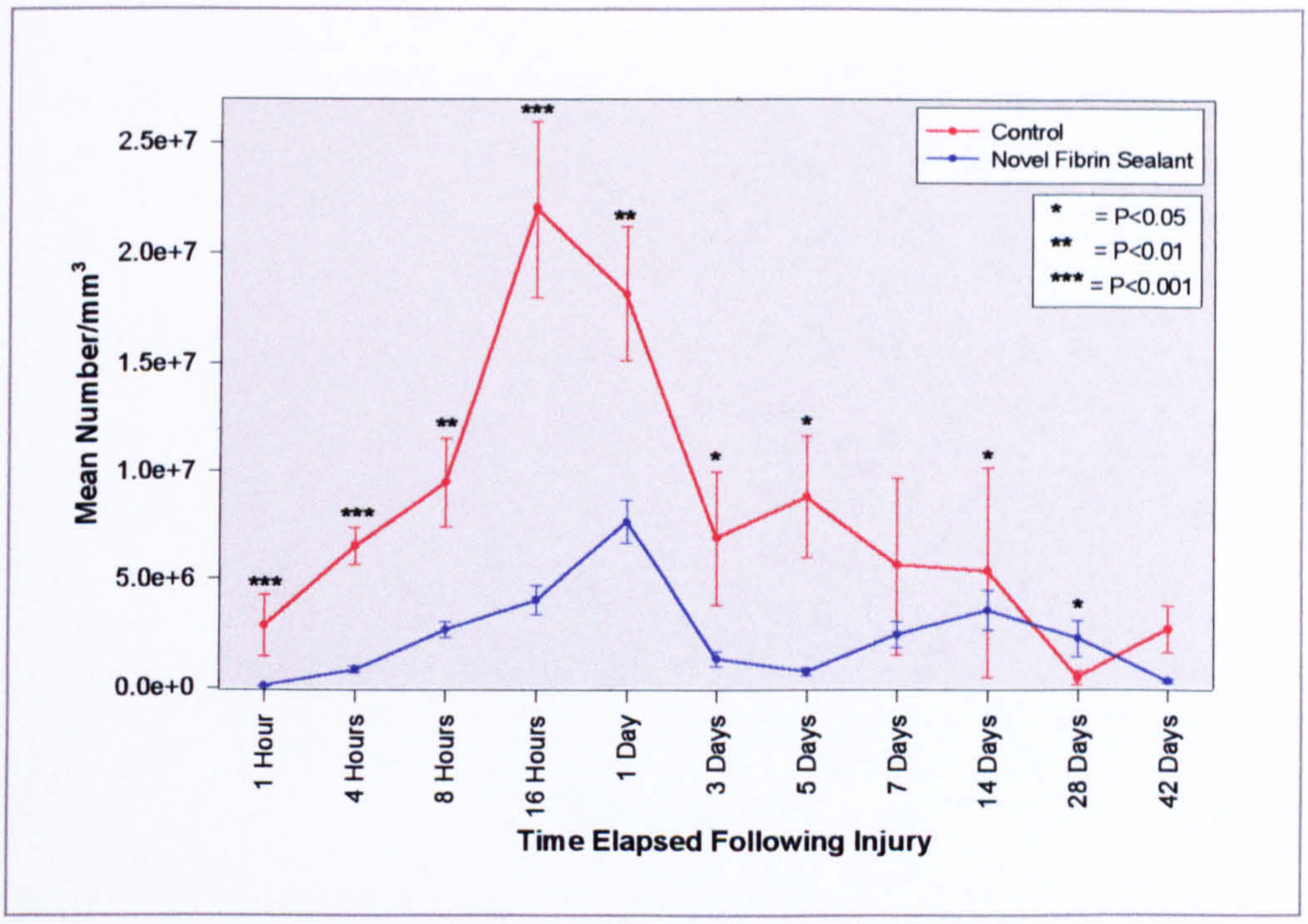
Control cases showed an increase in granulocyte mean numbers from 1 hour to peak at 16 hours with a rapid decrease to 3 days and a steady decline thereafter (Graph 5.24). Whereas treated cases showed an increase from 1 hour to 1 day, a rapid decrease from 1 to 3 days, a second increase from 3 to 14 days and decreasing numbers until the end of the study (Graph 5.24). At all time points, except 7, 28 and 42 days, mean numbers of granulocytes were significantly greater ($P<0.05$) in the control group compared to treated. Whereas at 28 days post injury significantly greater ($P<0.05$) mean numbers were present in treated cases compared to controls (Appendix V Table 5.4).

Similar trends were seen in both control and treated cases with regard to mean macrophage numbers (Graph 5.25). Minimal numbers were present from 1 hour to 1 day for control cases and 16 hours for treated cases. A rapid increase was seen from 1 to 5 days in control cases whereas a steady increase from 16 hours to 3 days followed by a rapid increase to 5 days was demonstrated by treated cases. Both groups peaked at 5 days and then decreased to 42 days. Significantly greater numbers ($P<0.05$) were present in control cases compared to treated at 1 hour and 3 days whereas at 1 and 28 days significantly greater numbers ($P<0.05$) were seen in treated compared to control cases (Appendix V Table 5.4).

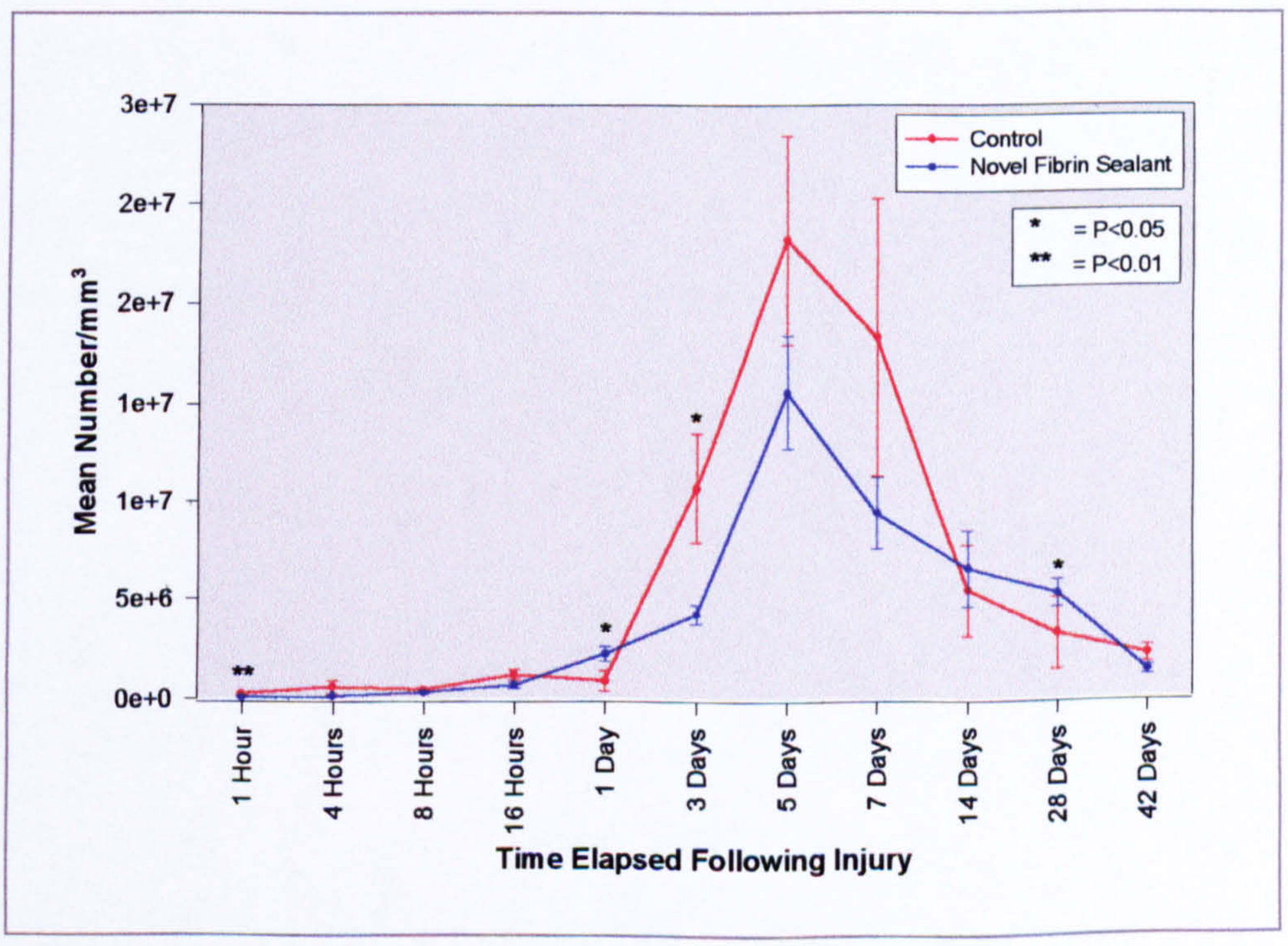
Control and treated cases showed the exact same trend for fibroblast mean numbers throughout the first 42 days of healing (Graph 5.26). Fibroblasts were first evident at 1 day and a slight increase was seen from this time point to 3 days. Mean numbers rapidly increased to peak at 5 days and a second peak at 14 days, with declining numbers for the remainder of the study. Control cases demonstrated significantly greater numbers ($P<0.05$) compared to treated cases at 4 hours and from 3 days onwards (Appendix V Table 5.4). Significantly higher numbers ($P<0.001$) were seen with treated cases at 1 day compared to controls.



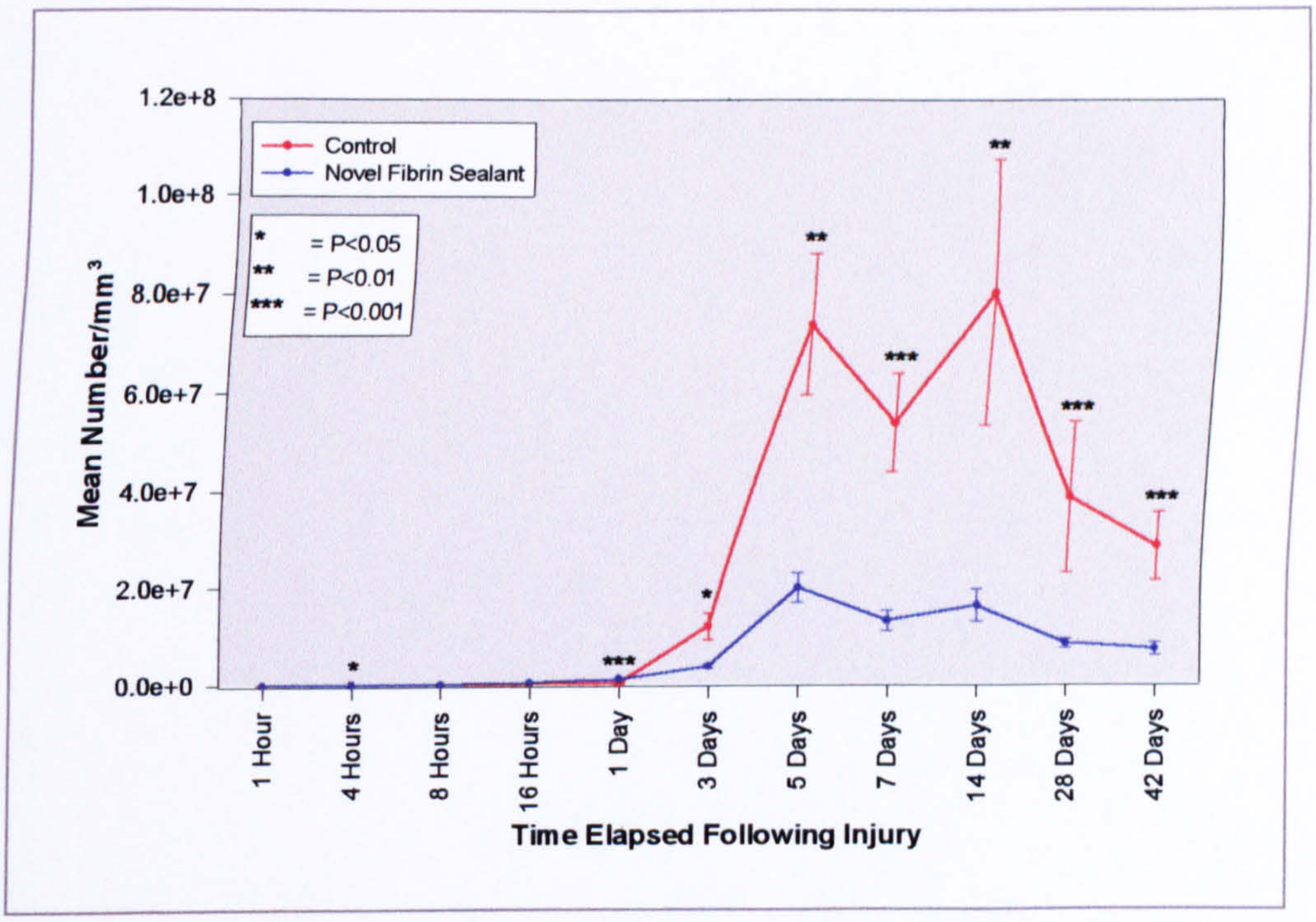
Graph 5.23: Mean total cell numbers throughout TTG development together with SEMs and statistical differences.



Graph 5.24: Mean granulocyte numbers throughout TTG development together with SEMs and statistical differences.

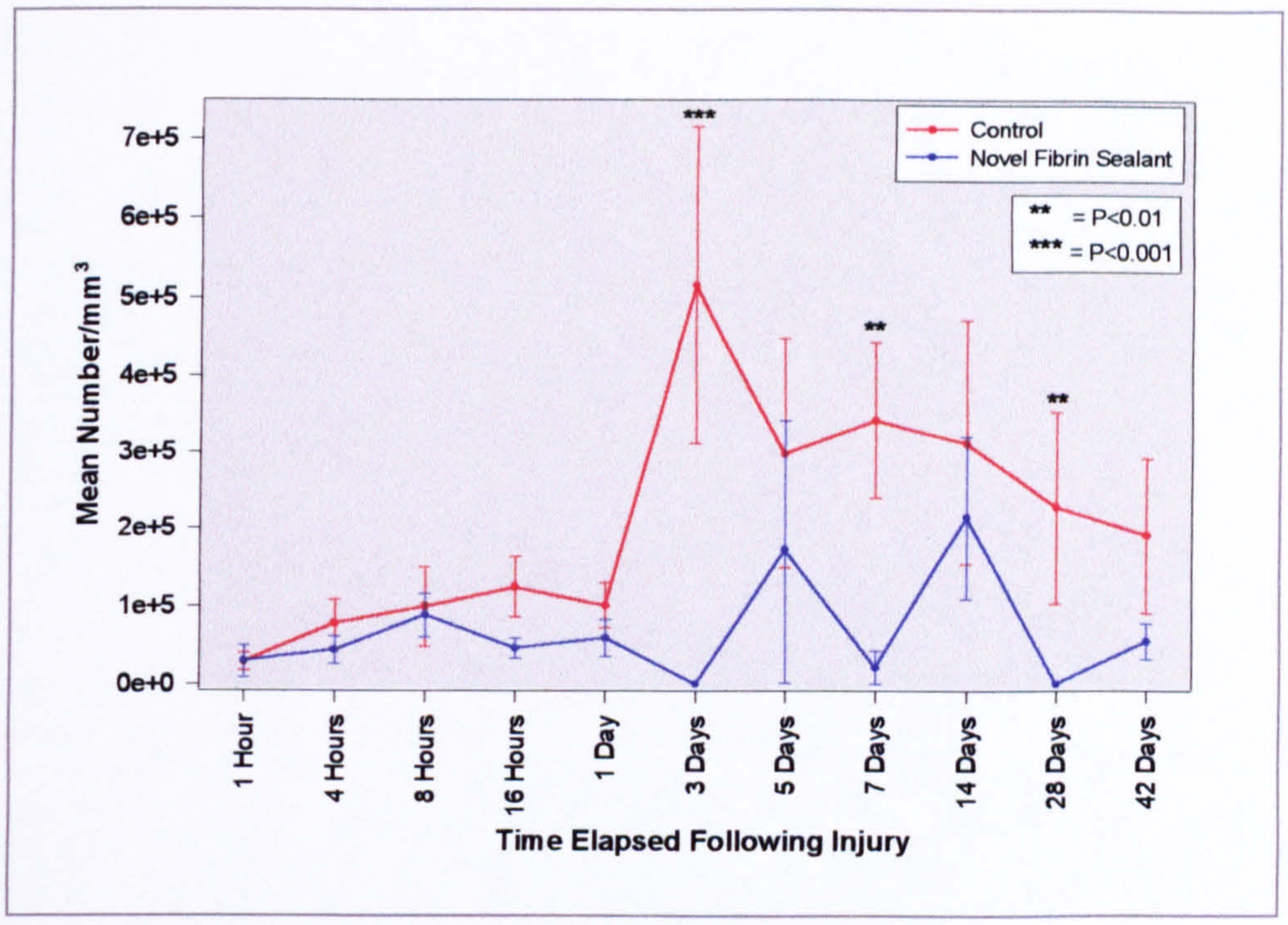


Graph 5.25: Mean macrophages numbers throughout TTG development together with SEMs and statistical differences.



Graph 5.26: Mean fibroblast numbers throughout TTG development together with SEMs and statistical differences.

Mean numbers of lymphocytes in control cases showed minimal numbers with little variation from 1 hour to 1 day post injury. From 1 to 3 days there was a sharp increase to peak with numbers decreasing thereafter (Graph 5.27). Treated cases were the same as control during the first day however from 3 to 28 days numbers fluctuated frequently. At all time points control cases demonstrated greater mean numbers of lymphocytes with this being significant ($P<0.01$) at 3, 7 and 28 days (Appendix V Table 5.4).



Graph 5.27: Mean lymphocyte numbers throughout TTG development together with SEMs and statistical differences.

5.4.2 Discussion

Control and novel fibrin sealant treated cases demonstrated the same trend for cell predominances within injury tissue. For the first day following injury, inflammatory cells (eosinophilic cells, basophilic cells, macrophages) were the predominant cell types and from 3 days onwards tissue repair cells were predominant (fibroblasts, macrophages). Hence treatment with novel fibrin sealant does not effect the overall cellular pattern of wound healing but it did exert subtle changes with the main modifications being the greater percentages of fibroblasts at 16 hours and 1 day and greater percentages of macrophages from 5 days onwards in treated cases compared to control cases. These will be discussed shortly.

Similar trends in cell densities and totals further substantiates that novel fibrin sealant does not alter the course of wound healing (Figure 5.3). Volumetric analysis of control and treated cases with regard to tissue zones found that total tissue generation was significantly greater in controls consequently it follows that all number totals were greater in control cases. This pattern was also found in all cellular densities except macrophages where cell densities were greater in treated cases. Therefore PSA

development attracts a greater cell presence compared to PSA prevention by novel fibrin sealant. Since cells present release cytokines and growth factors which, in turn, induce further cell recruitment and so forth, this reciprocal spirals and probably exacerbates wound healing and PSA development. Reduced cellular presence in novel fibrin sealant cases is indicative of chemical modulation or limitation due to either tissue isolation or via direct chemical activity by novel fibrin sealant.

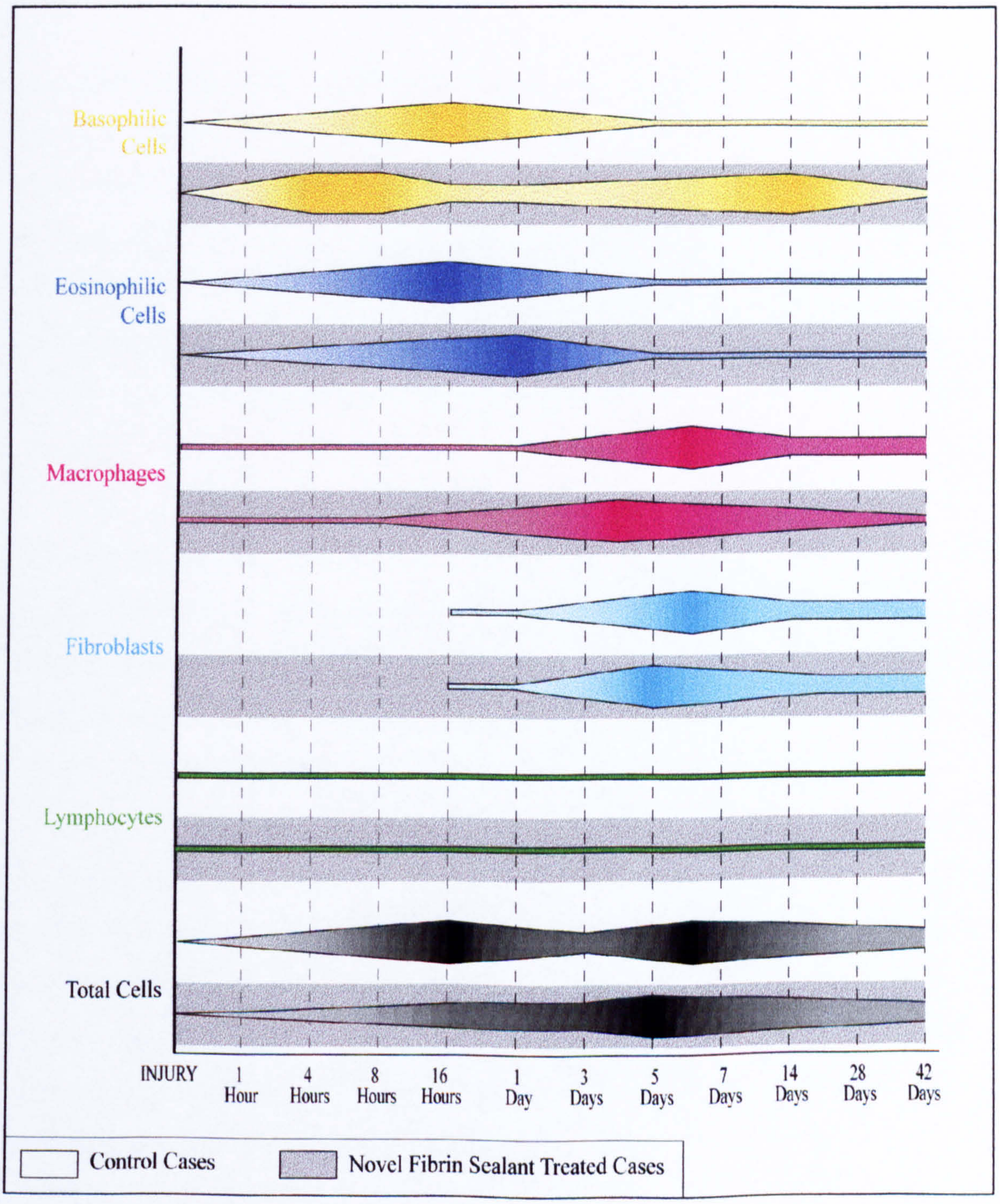


Figure 5.3: Summary of cellular changes.

At 1 hour post injury, greater total cell densities were demonstrated in controls compared to treated cases. We know that novel fibrin sealant is an effective haemostatic agent reducing the necessity for natural haemostatic mechanisms. Therefore treated cases will have reduced natural fibrin and platelet plug which in turn means less factors secreted by platelets present and, as a net result, reduced cellular recruitment to the site of injury. Even at 1 hour following injury the effects and benefits of novel fibrin sealant are clearly evident.

Peaks in total cell densities were seen at 16 hours for controls and 1 day for treated and 5 days for both groups. We know that from 1 to 16 hours PSA modelling is actively occurring with fibrin lay down in both control and treated cases. During this time inflammatory cells are predominant and these appear to play a major role in continual fibrin deposition. In contrast from 16 hours to 1 day onwards, PSA and tissue generation remodelling is occurring. The second peak in /cell densities at 5 days is directly related to the presence of tissue repair cells, especially fibroblasts synthesising collagen, within injury tissue.

The peak in granulocyte densities at 16 hours for control cases was much greater compared to the peak at 1 day for treated cases. Control cases experience a much greater inflammatory reaction to injury compared to treated cases. This is due to biolimitation by novel fibrin sealant with reduced inflammatory response having the knock on effects of reducing tissue necrosis and therefore reduced repair tissue required.

Macrophage densities were greater in treated compared to control cases, with increased phagocytic activity within treated cases. This is most probably due to the non-autologous nature of the fibrin sealant utilised in this experimental model since macrophages were the primary cell type seen in fibrin sealant associated cell concentration. Increased macrophages mean increased phagocytosis which has the effect of rapid necrotic tissue clearance and reduced time required for wound healing.

Significantly greater fibroblast densities were seen at 16 hours and 1 day post injury in

treated compared to control cases. This shows that the second stage of wound healing, tissue repair/remodelling is initiated earlier with novel fibrin sealant treatment. This is possibly due to reduced amount and time taken for the inflammatory reaction following injury, allowing tissue repair to be initiated earlier.

Lymphocytes were present in greater densities in treated cases compared to controls from 1 to 8 hours however this relationship was reversed at 3, 7 and 28 days post injury. It may be that the introduction of a foreign protein leads to a rapid lymphocyte mounted immune response. It is also possible that novel fibrin sealant has anti-bacterial properties or that with limited inflammation and consequent necrosis there is a reduced lymphocyte response.

Conclusions

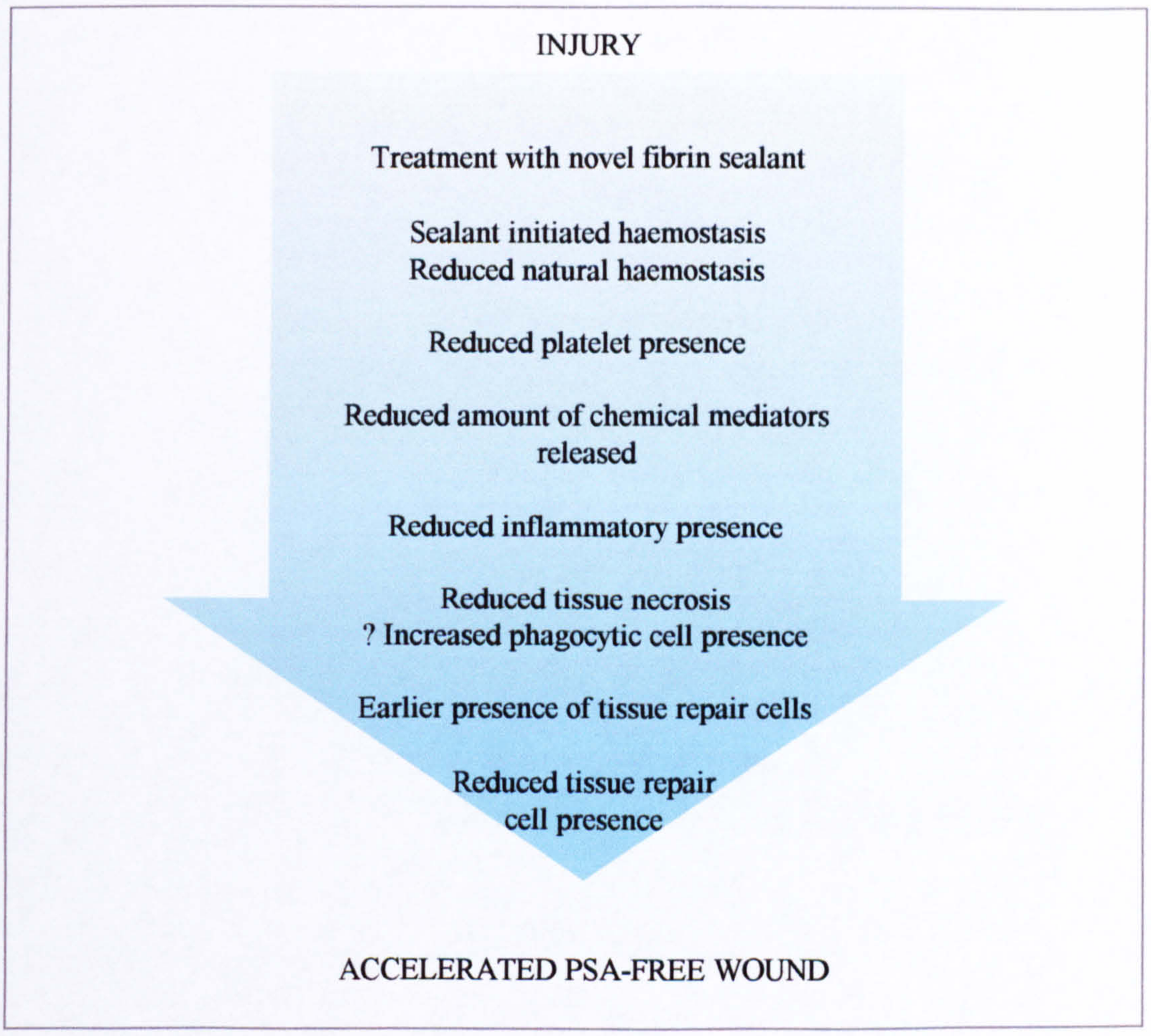
i. Treatment with novel fibrin sealant does not alter the pattern of cellular involvement in wound healing.

ii. Treatment with novel fibrin sealant does reduce both densities and numbers of eosinophilic cells, basophilic cells, fibroblasts, lymphocytes and macrophages (number totals only) present in injury tissue from a very early stage of wound healing (1 hour or maybe earlier post injury).

iii. Greater macrophage densities with novel fibrin sealant in this study could be due to:

- introduction of a foreign protein
- actual effect of treatment with novel fibrin sealant

iv. Treatment with novel fibrin sealant does exert alteration on wound healing as follows:



5.5 Biochemical Analyses

5.5.1 Full and Differential Blood Counts

In an attempt to identify systemic changes occurring with the use of novel fibrin sealant, full and differential blood counts from animals within control and treated groups will be compared.

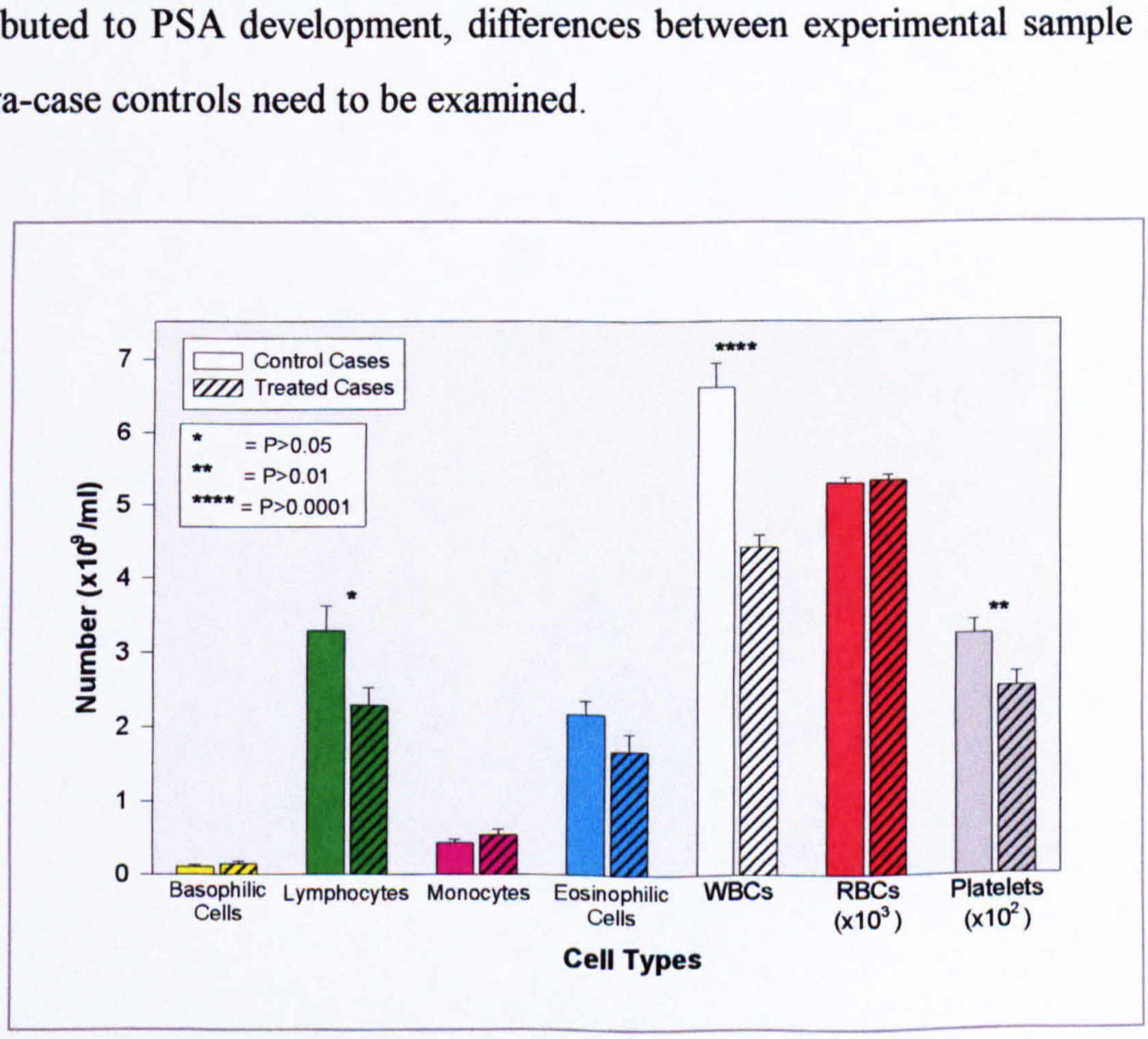
5.5.1.1 Results

Full and differential blood counts for control and treated cases at blood sample 1 demonstrated significant differences for between the two groups (Graph 5.28; Appendix V Table 5.5). The control group had significantly greater mean numbers of WBCs ($P<0.001$), lymphocytes ($P<0.05$) and platelets ($P<0.01$) compared to the treated group.

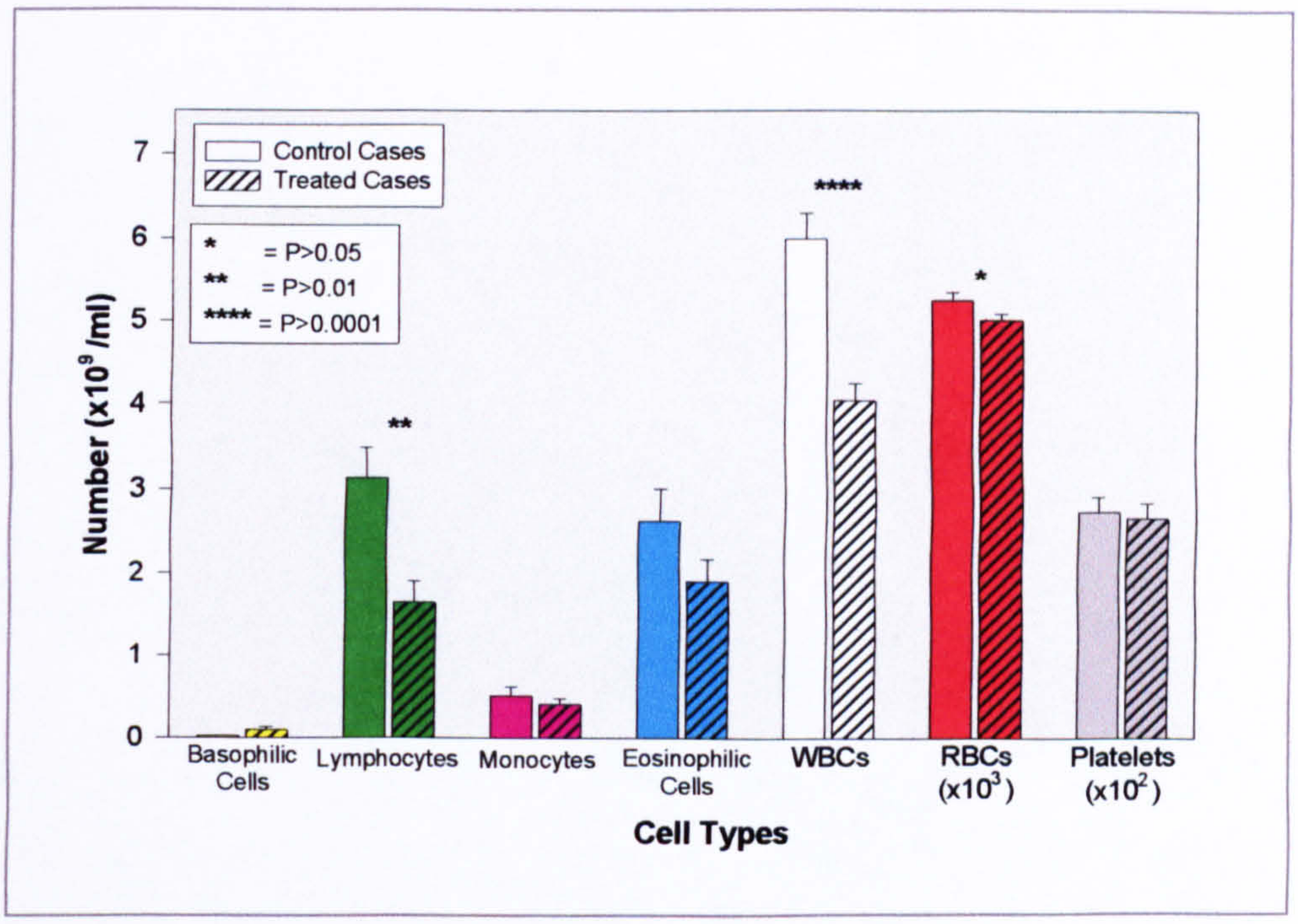
Similar differences were present between control and treated samples at blood sample 2, with the control group having significantly greater mean numbers of WBCs ($P<0.001$), lymphocytes ($P<0.01$) and RBCs ($P<0.05$) (Graph 5.29; Appendix V Table 5.5).

At blood sample 3 significant differences between control and treated cases were also present, with the control group demonstrated significantly greater mean numbers of WBCs ($P<0.001$), lymphocytes ($P<0.001$) and eosinophilic cells ($P<0.05$) (Graph 5.30; Appendix V Table 5.5).

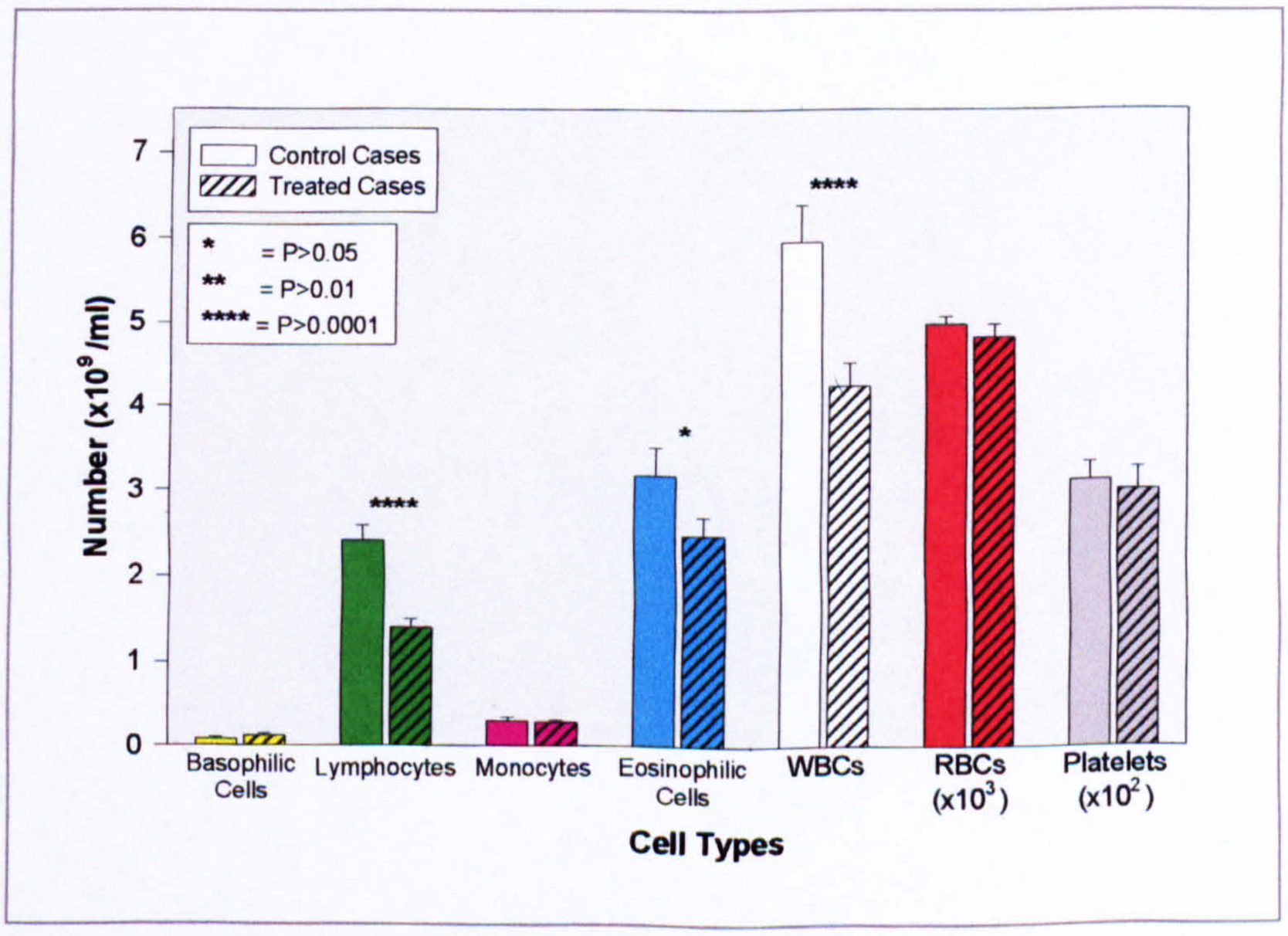
Since blood samples 1 and 2 are resting controls and post-laparotomy controls respectively, significant differences between control and treated groups mean direct comparison between experimental blood sample 3 for the groups is invalid. To determine whether novel fibrin sealant is exerting any systemic effects which are unique to itself and not attributed to PSA development, differences between experimental sample 3 and its own intra-case controls need to be examined.



Graph 5.28: Full and differential blood counts at blood sample 1 together with SEMs and statistical differences.



Graph 5.29: Full and differential blood counts at blood sample 2 together with SEMs and statistical differences.

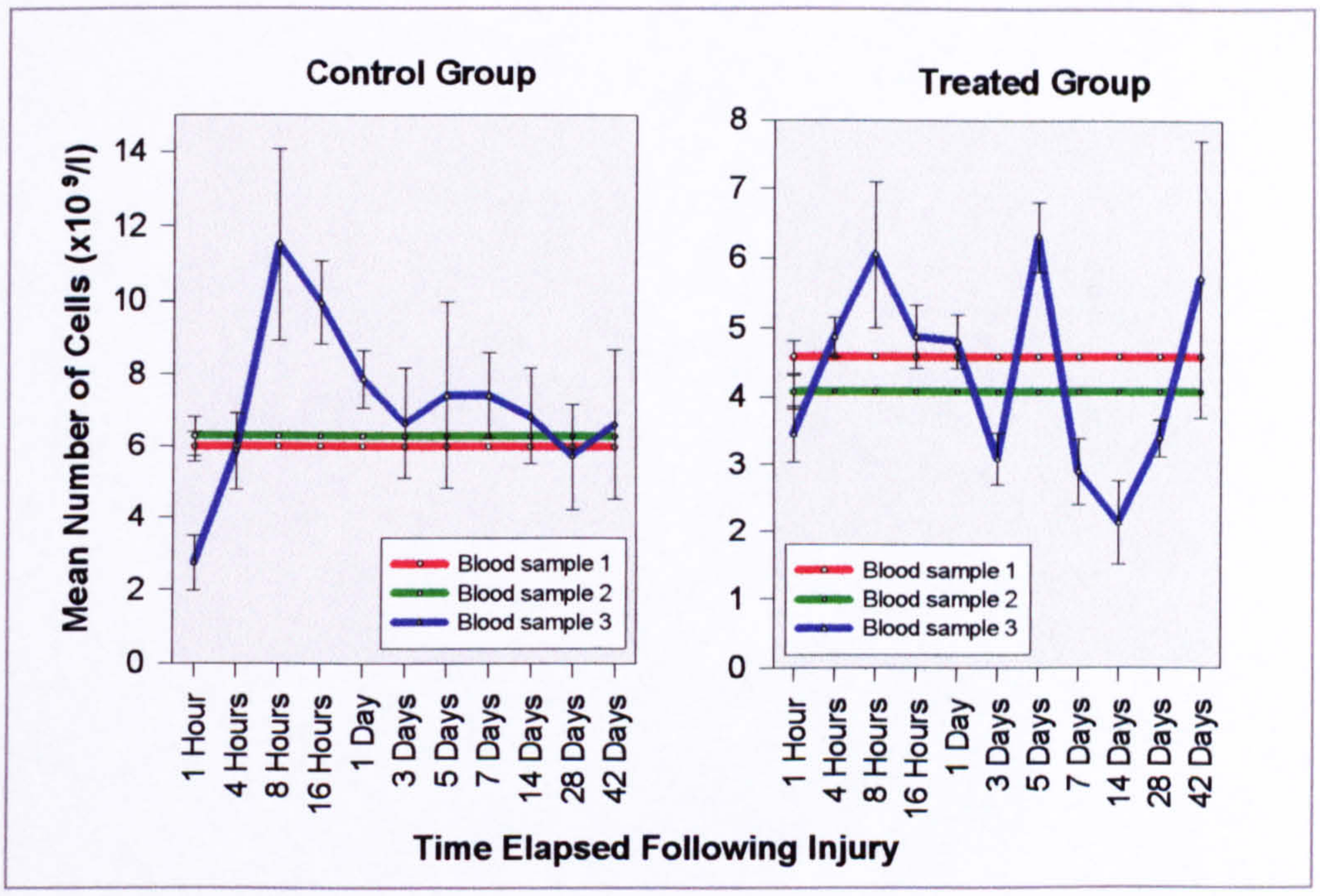


Graph 5.30: Full and differential blood counts at blood sample 3 together with SEMs and statistical differences.

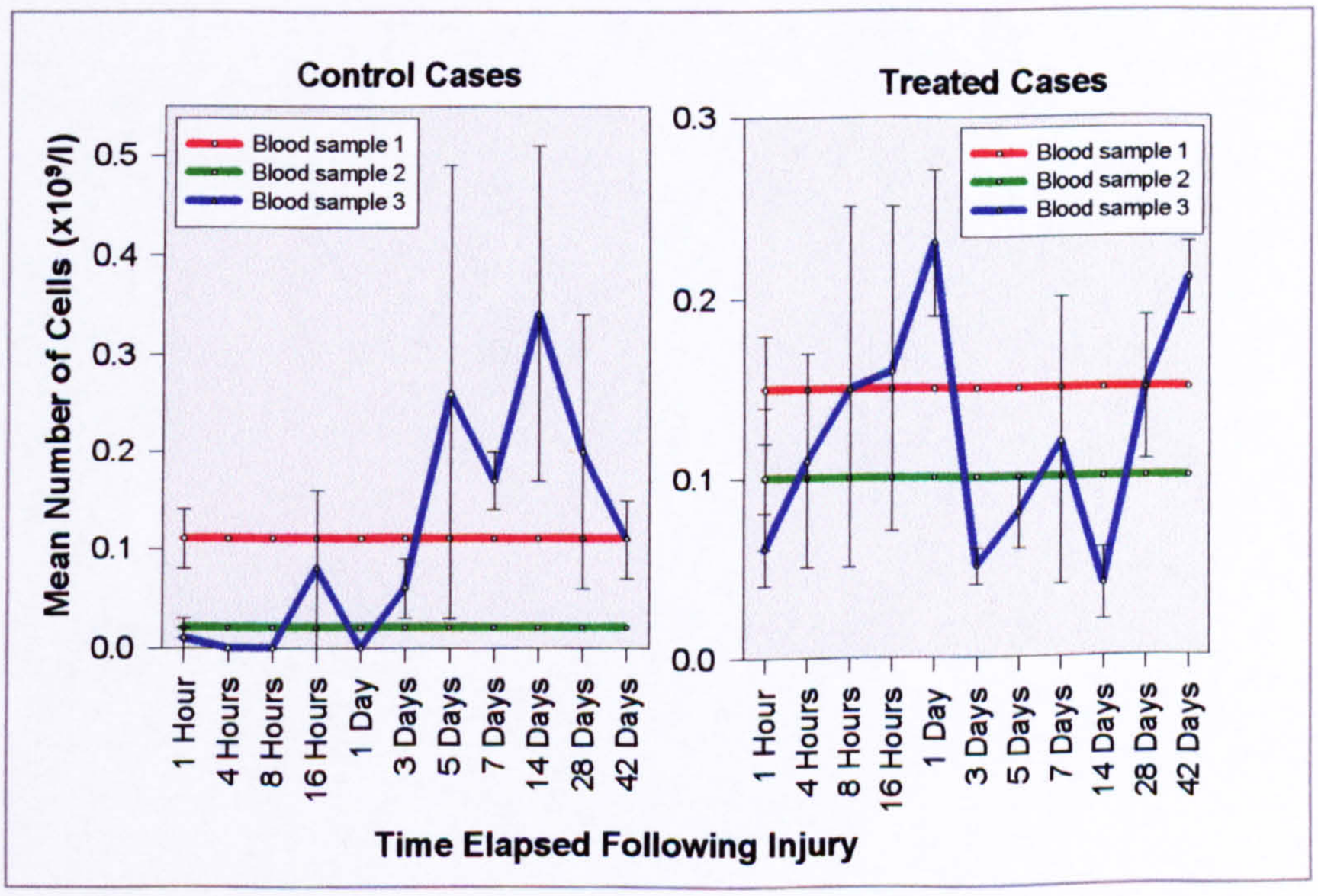
RBCs and platelet mean numbers at experimental blood sample 3 demonstrated no significant differences from blood samples 1 or 2 in either control or treated group.

Mean numbers of WBCs in blood sample 3 did demonstrated differing trends for control and treated cases (Graph 5.31). In control cases mean numbers increased from 1 hour to peak at 8 hours and then decreased to intra-case control values by 3 days, with a minor second peak at 5 to 7 days. Whereas treated cases also increased from 1 hour to peak at 8 hours and then decreased to 3 days to drop below intra-case control levels. A second peak was seen at 5 days with a rapid decrease to 14 days, with levels returning to intra-case control values by the end of the study.

Basophilic cell mean numbers showed different trends for control and treated groups (Graph 5.32). In control cases mean numbers remained low for the first day after which they increased to peak at 5 and then 7 days, with values returning to intra-case controls by 42 days. Treated cases demonstrated mean numbers increasing from 1 hour to peak at 1 day, with a rapid decrease from 3 to 14 days and values rising to 42 days post injury.



Graph 5.31: Mean numbers of WBCs for all blood samples together with SEMs.

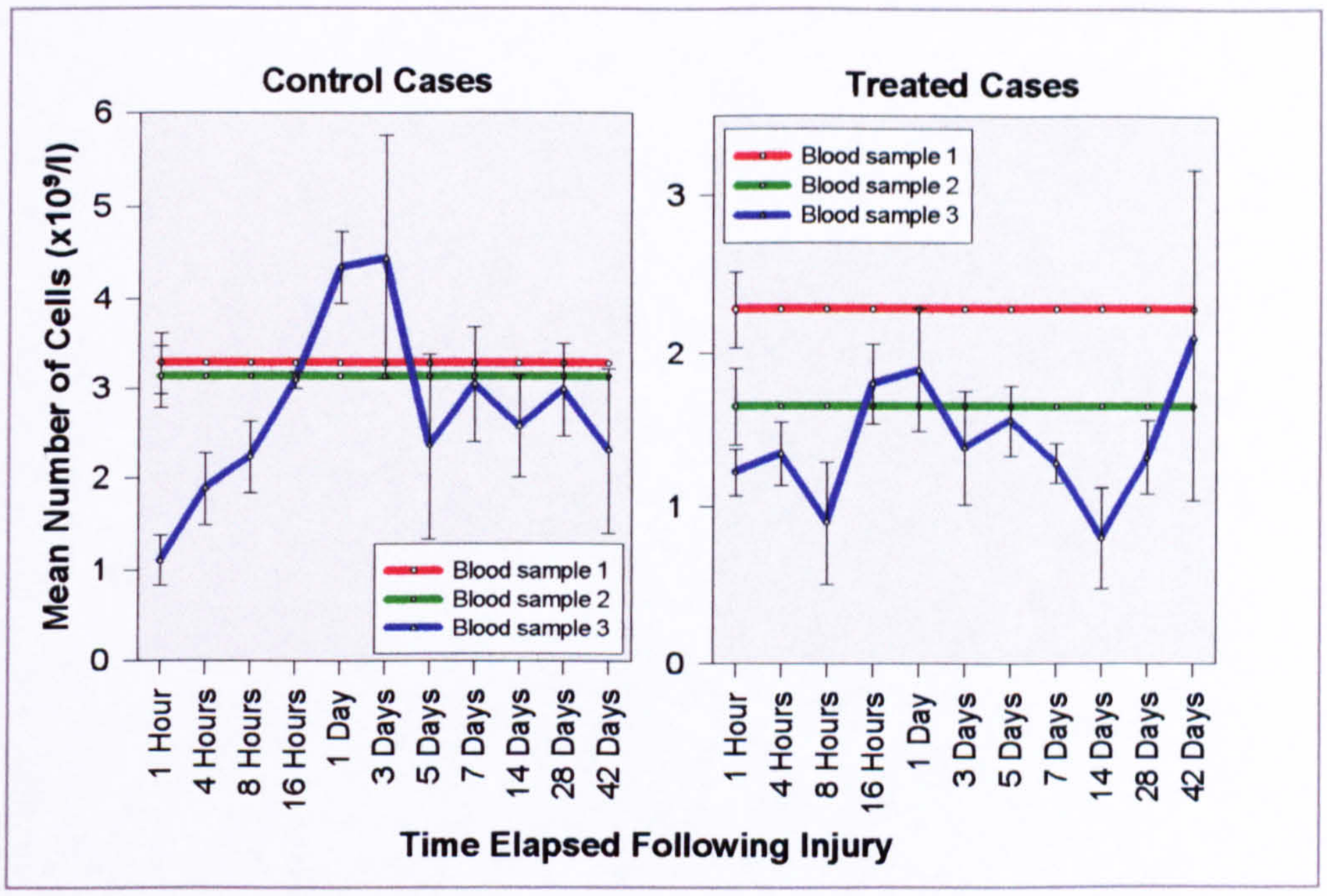


Graph 5.32: Mean numbers of basophilic cells for all blood samples together with SEMs.

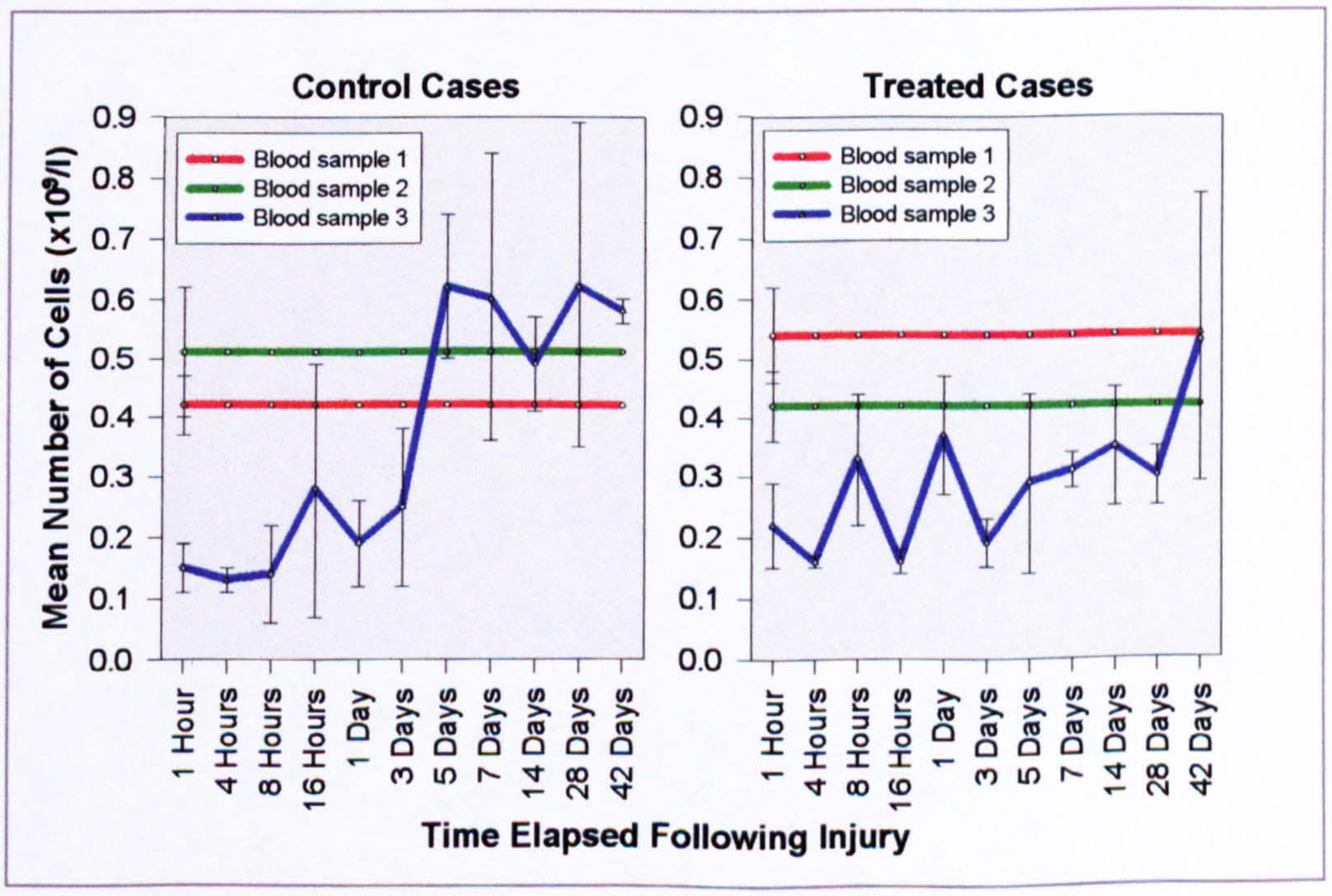
Control and treated groups demonstrated different trends in cell numbers with regard to lymphocytes (Graph 5.33). Mean numbers increased from 1 hour to peak at 1 to 3 days in control cases, with values decreasing to just below intra-case controls for the remainder of the study. However treated cases showed little change in lymphocyte mean numbers from 1 to 8 hours, after which there was a peak at 16 hours and 1 day, with levels decreasing slightly to 14 days and then returning to intra-case control mean numbers.

Control and treated groups also demonstrated different patterns for mean monocyte numbers throughout this study (Graph 5.34). From 1 hour to 3 days mean numbers for control cases remained below intra-case controls, however they increased rapidly in intra-case controls at 5 days with values remaining similar to 42 days. Treated cases on the other hand showed little variation from 1 hour to 42 days, with mean numbers below intra-case controls.

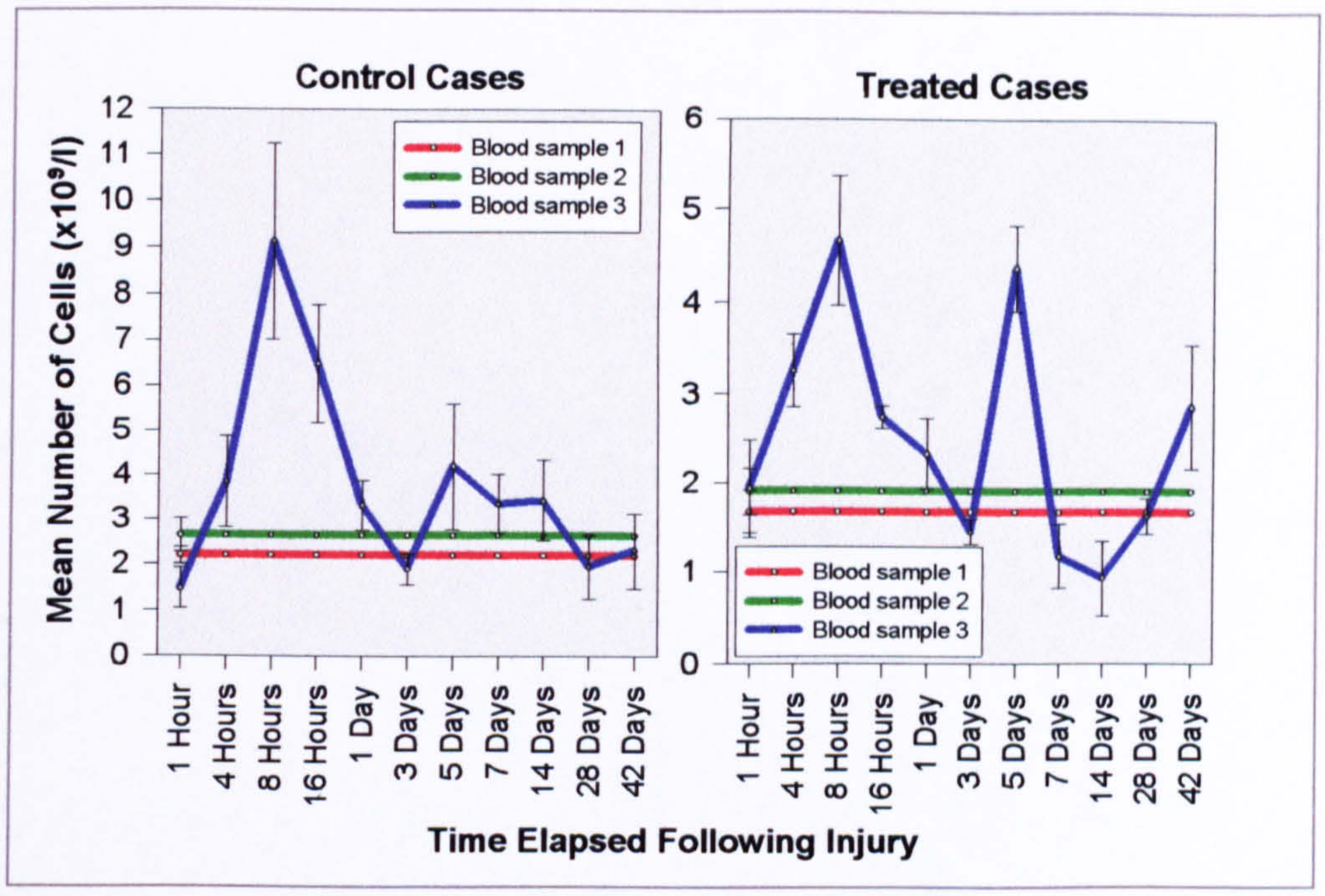
Both control and treated cases demonstrated a similar trend for mean numbers of eosinophilic cells (Graph 5.35). Both showed increases in mean numbers of eosinophilic cells from 1 hour to peak at 8 hours. This was followed by a rapid decrease to 3 days after which mean numbers increased to peak for a second time at 5 days post injury. Mean eosinophilic cell numbers then decreased steadily in control cases and rapidly for treated cases with levels settling out by the end of the study.



Graph 5.33: Mean numbers of lymphocytes for all blood samples together with SEMs.



Graph 5.34: Mean numbers of monocytes for all blood samples together with SEMs.



Graph 5.35: Mean numbers of eosinophilic cells for all blood samples together with SEMs.

5.5.1.2 Discussion

Blood samples 1 and 2 were taken to act as intra-case controls at resting and post-laparotomy respectively. Although full and differential blood counts at blood samples 1 and 2 for control and treated groups fell within normal values for New Zealand White rabbits they were significantly different between groups. This made direct comparisons between control and treated groups, that is, PSA development and prevention with novel fibrin sealant respectively, of blood counts at blood sample 3 invalid. Hence the only way to identify changes associated with either PSA development or prevention was to compare differences with intra-case controls. Since PSA development and PSA prevention with novel fibrin sealant were different studies, they were performed at different times, with animals admitted to the department at different times. It is most probable that animals obtained were from different breeding lines, which would account for slightly different haematological base levels.

Neither PSA development or PSA prevention with novel fibrin sealant had any notable effect on mean numbers of RBCs or platelets during the course of this study. Since these cells do not play major roles in wound healing following haemostasis these results are as

expected.

WBCs on the other hand are heavily involved in both PSA development and PSA prevention with novel fibrin sealant and the data obtained during this study demonstrates that these two processes do produce differing effects on systemic cell numbers. However care needs to be taken when interpreting this data due to the effects of venesection on cell numbers. Chapters 3 and 4 both detail whether changes in cell numbers at blood sample 3 are due to venesection or PSA development/prevention with novel fibrin sealant respectively. Table 5.4 summaries cell number changes and identifies their probable cause.

Group	Cell Type	Changes Associated with:	
		Venesection	**PSA Development or Prevention**
Control	WBCs	Changes from 16 hours onwards	Increased numbers from 1 to 8 hours
	Basophilic Cells	Changes up to 7 days	Elevated levels at 14 and 28 days
	Lymphocytes	All changes	-
	Monocytes	-	Reduced numbers from 1 hour to 3 days
	Eosinophilic Cells	-	Peak at 8 hours
Novel Fibrin Sealant Treated	WBCs	Undetermined effect	Increased numbers from 1 hour to 1 day ? peak at 5 days
	Basophilic Cells	All changes	-
	Lymphocytes	All changes	-
	Monocytes	-	Lower mean numbers throughout the study
	Eosinophilic Cells	-	Peaks at 8 hours and 5 days

Table 5.4: Changes in WBC numbers associated with venesection and PSA development or prevention with novel fibrin sealant.

Both PSA development and treatment with novel fibrin sealant increased mean numbers of WBCs from 1 hour to 8 hours and 1 day respectively. Following injury there is a transient elevation in mean numbers of circulating WBCs whether injury sites are treated or not due to mobilisation of extra WBCs from storage tissues. In addition a peak in WBC numbers was seen at 5 days post injury for the novel fibrin sealant treated cases. It

is hypothesised that this is due to the immune reaction initiated by the presence of human fibrin in the rabbit peritoneum and in the proposed system for novel fibrin sealant (human: human) this systemic reaction would not be present.

PSA development did exert an effect on basophilic cells, with elevated numbers circulating at 14 and to a lesser extent 28 days following injury. No effect was seen in the novel fibrin sealant treated group. It is probable that these elevated numbers at the later stages of PSA development are due the sustained reaction induced by PSA formation. In treated cases wound healing is completed at an accelerated rate with reduced reaction and necrotic damage, therefore these later systemic effects are absent in the treated group.

Neither PSA development nor prevention with novel fibrin sealant demonstrated noticeable effects on systemic lymphocyte numbers. Initially lower levels in both groups can be directly attributed to venesection and lower circulating levels from 3 days onwards in the treated group is probably due to biological variation. No infection was seen in any of the animals in this study and although fibrin sealant induced an immune response it did not attract great quantities of lymphocytes.

PSA development produced reduced numbers of monocytes from 1 hour to 3 days post injury. It is hypothesised that this is due to the large role played by these phagocytic cells during inflammation and tissue repair, with monocytes leaving the circulatory system and entering tissues. A similar effect is also seen in the treated group although to a lesser degree. This corresponds with the reduced inflammatory reaction seen with novel fibrin sealant treatment.

Both PSA development and treatment with novel fibrin sealant resulted in a peak in circulating eosinophilic cells at 8 hours post injury. The involvement of eosinophilic cells in PSA development is well documented, with these inflammatory cells attracted to the site of injury to be predominant at 16 hours post injury (Chapters 3, 4 and 5 of this thesis). It appears that additional eosinophilic cells are recruited into the circulatory

system to meet the high tissue demand. An additional peak in numbers was seen at 5 days for the treated group which is due to the immune mediated reaction to the presence of human blood derived novel fibrin sealant in this rabbit model.

Throughout this study cell numbers for PSA development demonstrated a greater range of variation compared to prevention with novel fibrin sealant. It is well established by the studies contained in this thesis that treatment with novel fibrin sealant limits the reaction mounted to injury and it appears that this local limitation has the effect of reducing systemic changes.

Conclusions

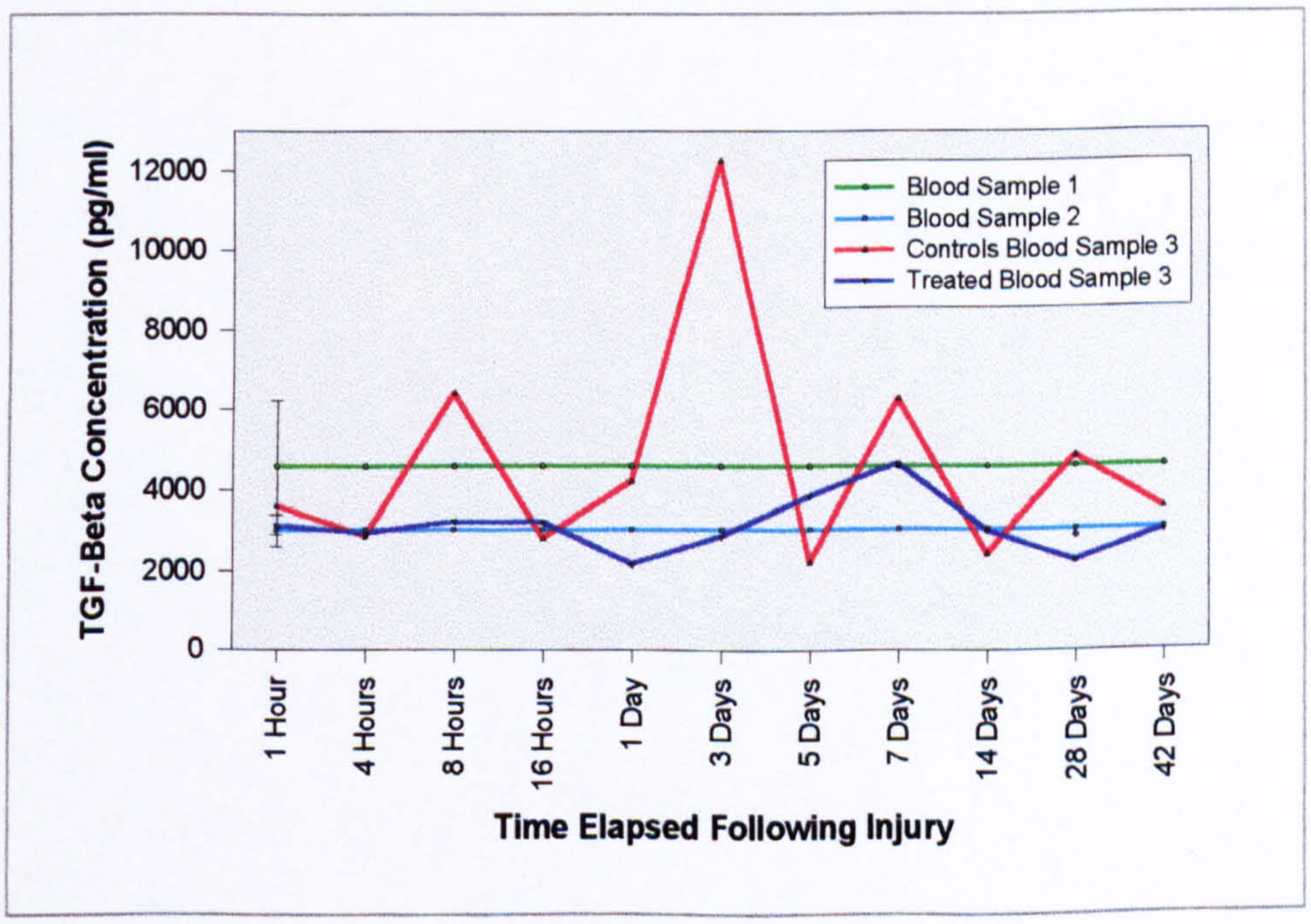
i. PSA development or PSA prevention with novel fibrin sealant:
    a. Had no notable effect on mean numbers of :
        - RBCs
        - Platelets
        - Lymphocytes
    b. Increased mean numbers of WBCs from 1 hour to 8 hours and 1 day respectively.
    c. Reduced mean numbers of monocytes from 1 hour to 3 days post injury (to a lesser degree in the treated group)
    d. Resulted in a peak in circulating eosinophilic cells at 8 hours post injury
ii. PSA development did exert an effect on basophilic cells, with elevated numbers circulating at 14 and to a lesser extant 28 days following injury. No effect was seen in the novel fibrin sealant treated group.
iii. Treatment with novel fibrin sealant caused an additional peak in eosinophilic cell and WBC mean numbers at 5 days due to the immune mediated reaction to the presence of human novel fibrin sealant in this rabbit model.
iv. PSA development demonstrated a greater range of variation of cell numbers compared to prevention with novel fibrin sealant.

5.5.2 TGF-β Concentrations

TGF-β concentration in plasma and peritoneal fluid samples collected throughout these studies were determined via an ELISA kit from R&D Systems. Due to time limitation only one plasma sample from 1 hour to 42 days was assayed for each group and one undiluted peritoneal fluid sample from 3 to 28 days together with controls.

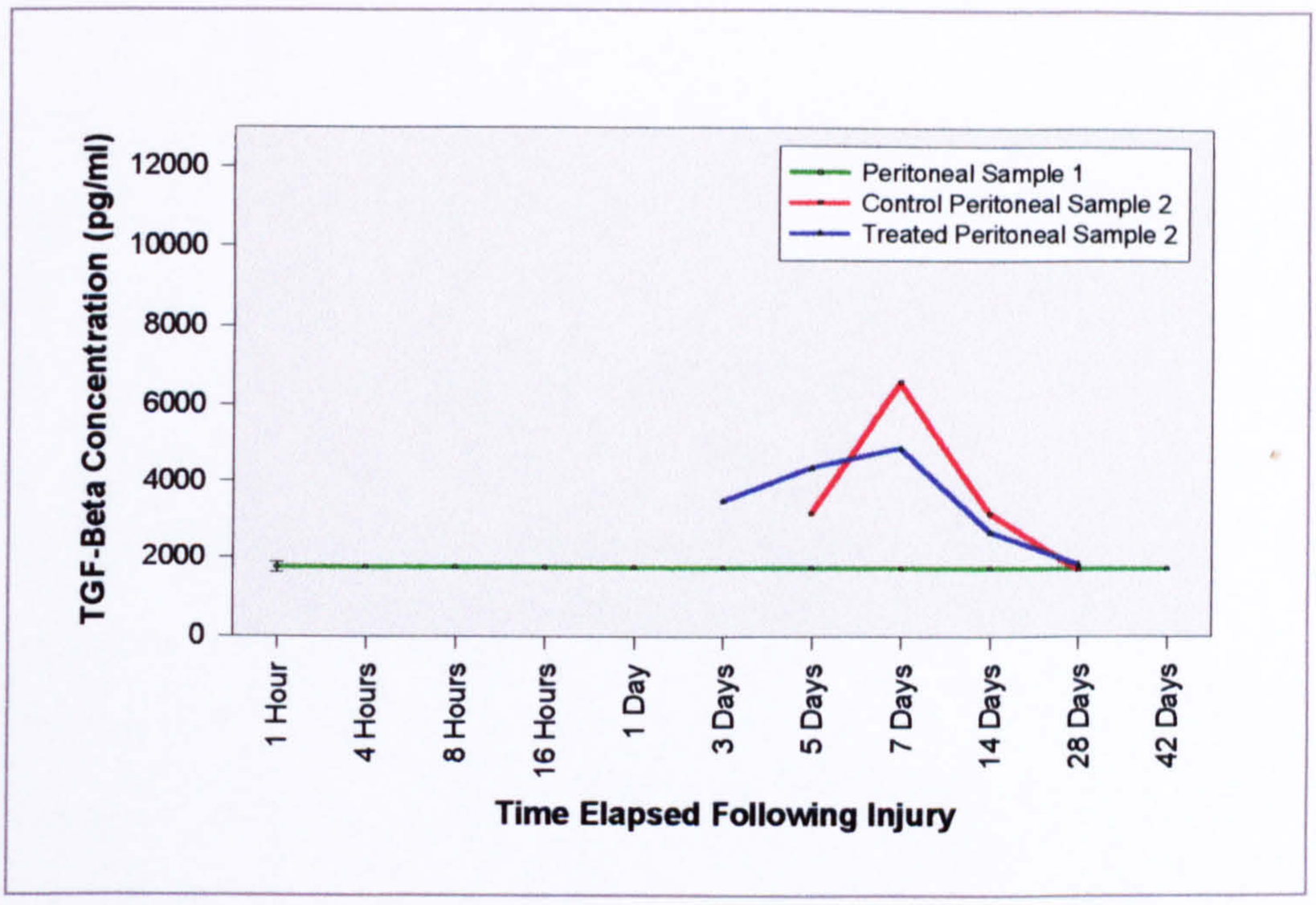
5.5.2.1 Results

Comparison of blood samples 3 from control and treated cases showed that control cases demonstrated greater fluctuations with regard to TGF-β concentrations (Graph 5.36). All treated samples were similar to normal levels but, although most control samples were within these limits, a large peak in TGF-β was seen at 3 days post injury.



Graph 5.36: Comparison of TGF-β concentrations present in blood samples.

With regard to TGF-β concentrations within peritoneal fluid samples, control and treated cases demonstrated a similar trend (Graph 5.37). Maximum concentration was seen at 7 days with levels decreasing to normal values by 28 days.



Graph 5.37: Comparison of TGF-β concentrations present in peritoneal fluid samples.

5.5.2.2 Discussion

Since only one sample from each time point was assayed for each group it is invalid to draw real conclusions from this study. Further analyses are required.

It does appear from the results obtained so far that PSA development exerts an effect on systemic levels of TGF-β whereas treatment with novel fibrin sealant does not. Throughout this study it has been demonstrated that treatment with novel fibrin sealant reduces the level of inflammation associated with injury, with decreased tissue necrosis and repair tissue generated. Hence the lack of systemic elevation of TGF-β in treated samples correlates with reduced tissue response to injury.

TGF-β concentration within peritoneal fluid were elevated to similar levels and at the same time points in both control and treated samples and suggests that the limitation effect by novel fibrin sealant treatment is less pronounced locally. Since treated sites are covered with the protective layer of fibrin sealant, exposure to peritoneal fluid and contents will be reduced compared to uncovered controls. Hence even if similar concentrations TGF-β are present within peritoneal fluid of treated cases, the actions of these chemical factors on cells present within tissue generation may be curbed.

Conclusions

i. Insufficient data is available at present to make definitive conclusions concerning the effects of PSA development and prevention with novel fibrin sealant on TGF-β concentration within plasma and peritoneal fluid.

ii. Preliminary data suggests:

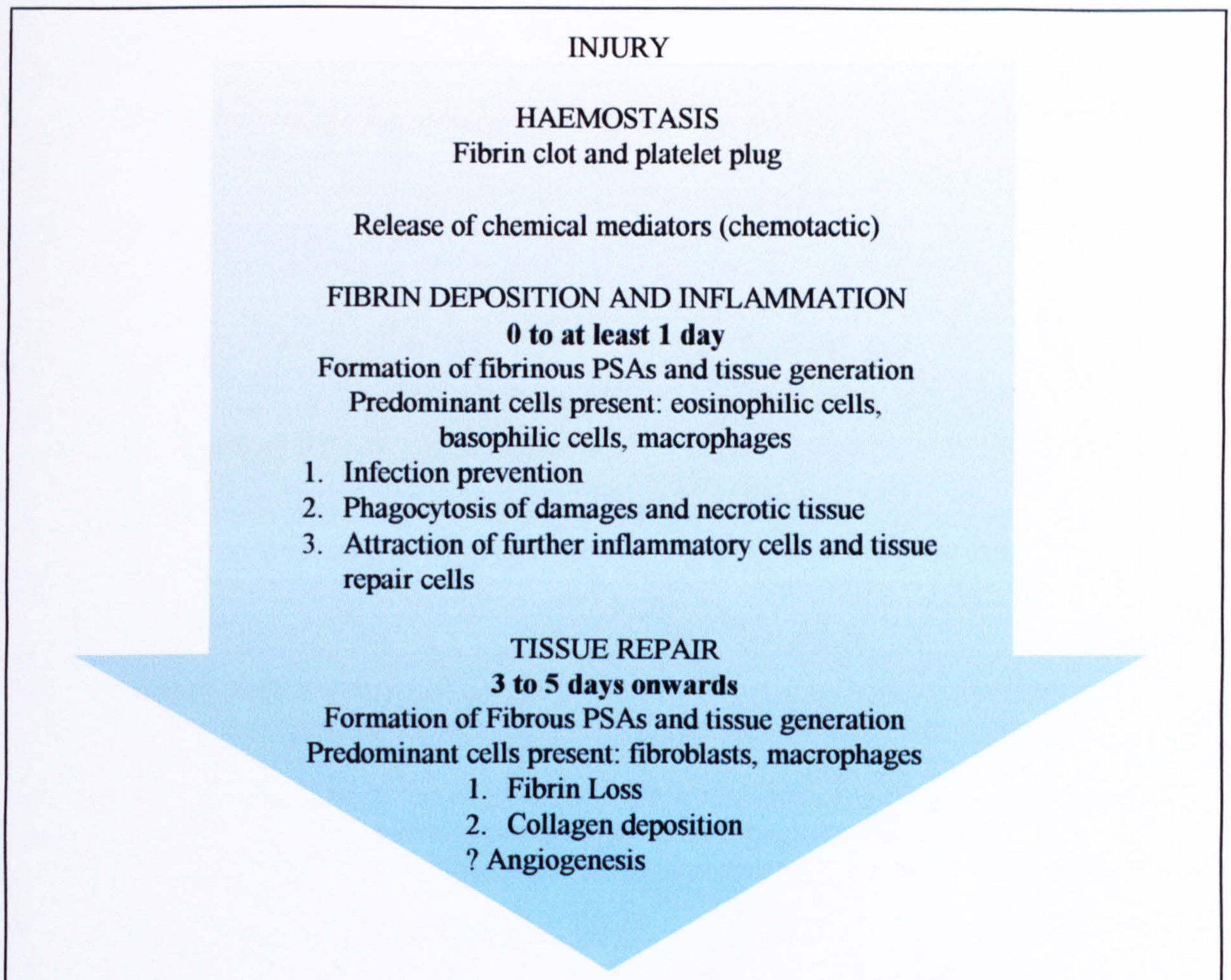
- PSA development elevated systemic TGF-β concentration in blood at 3 days and in peritoneal fluid at 7 days post injury.
- Treatment with novel fibrin sealant has no effect on blood TGF-β concentration but elevates peritoneal fluid concentrations at 7 days post injury.

5.6 Conclusions

Volumetric Conclusions

i. In this rabbit uterine horn abrasion model, treatment with novel fibrin sealant prevented PSAs formation in 96% of cases and decreased the volume and hence severity of PSAs by 99%.

ii. PSA development and prevention with novel fibrin sealant demonstrated the same pattern with regard to physiological, structural, cellular (local and systemic) and probably biochemical mechanisms.



iii. Novel fibrin sealant decreased the extent of changes and subsequently the time taken for those changes to occur:

- decreased volume of ischaemic and necrotic damage
- decreased volume of repair tissue generated
- decreased volume of peritoneal reaction
- accelerated maximum fibrin presence and fibrin loss
- accelerated collagen synthesis and maturation
- reduced densities and numbers of eosinophilic cells, basophilic cells, fibroblasts, lymphocytes and macrophages (number totals only) present in injury tissue from a very early stage of wound healing (1 hour or may earlier post injury).
- eliminated elevated numbers of circulating basophilic cells at 14 and 28 days following injury seen with PSA development.
- decreased magnitude of systemic cellular number changes compared to PSA

development

iv. Greater macrophages densities with novel fibrin sealant in this study could be due to:

- introduction of a foreign protein
- actual effect of treatment with novel fibrin sealant

v. Caused an additional peak in systemic numbers of eosinophilic cell and WBC mean numbers at 5 days due to the immune mediated reaction to the presence of human novel fibrin sealant in this rabbit model.

vi. Mechanisms of action for PSA prevention and alterations of wound healing by novel fibrin sealant include:

a. sealant acting as a haemostat and physical barrier, preventing fibrin exudate from joining injured surfaces and in turn preventing interactions between surfaces and peritoneal contents
b. biochemical modulations by the sealant.

6.0 Comparison of Preparation and Application Methods of Vivostat™ System Human Derived Fibrin Sealant for The Prevention of PSAs

6.1 Introduction

Previous studies, some of which are reported in this thesis (refer to Chapter 4), have shown that Vivostat™ system human derived fibrin sealant (novel fibrin sealant) significantly reduces and nearly completely prevents the incidence of PSAs in a definitive rabbit uterine horn abrasion model. All of these studies used automated preparation and application techniques (Vivostat™ processor and spray applicator) (refer to Section 2.8.1).

During early product development stages of novel fibrin sealant, preparation of the sealant was performed manually. Although this preparation technique was relatively easy and quick for scientists, it was non-viable for a commercial surgical product. Hence the fully automated Vivostat™ processor (Section 2.8.1) was introduced, using the same biochemical processes but with minimal human input and decreased preparation time. Since these two preparation methods utilise the same chemical processes the resulting fibrin sealant should be analogous, however fibrin sealant produced via manual and automated techniques has not been compared for PSA prevention to date.

Technical developments have also occurred with regard to application systems for novel fibrin sealant. Although the Vivostat™ automated spray applicator (Section 2.8.1) is the system of choice, several different systems have been investigated for sealant application, the main alternatives being:

i. UNES gun - a manual alternative to the device applicator comprising of a dual syringe system, based on the Duploject system (Figure 1.12). As with the device applicator, one syringe contains fibrin I solution and the other carbonate/bicarbonate buffer (pH 10.0). Syringes are mounted into a casing where depression of the trigger releases the contents of the two syringes in appropriate ratios, mixing taking place in

the Y-Piece where repolymerisation of fibrin I is initiated, with the resultant fibrin II sealant ejected from the attached needle (Figure 6.1).

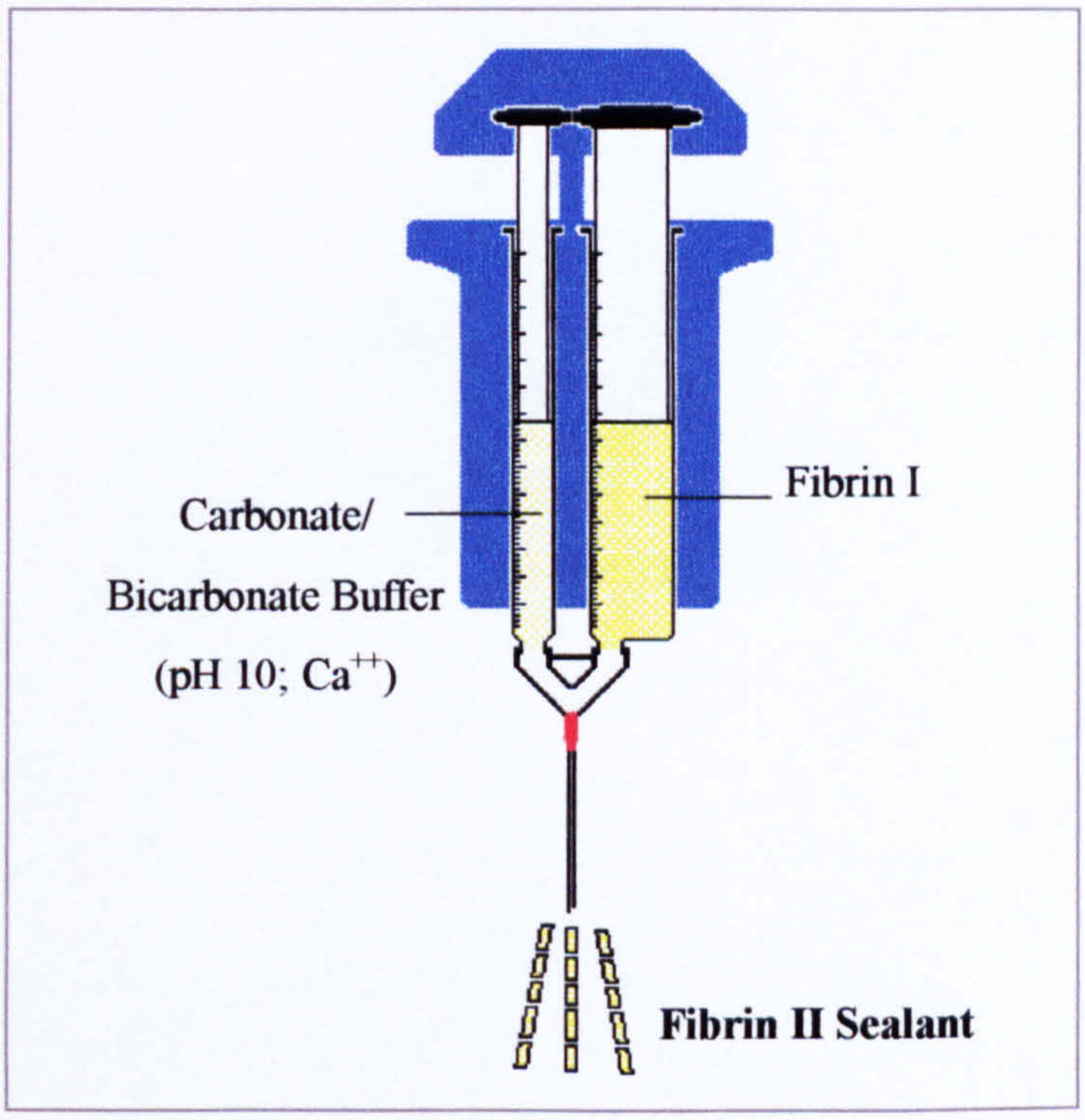


Figure 6.1: UNES gun Vivostat™ system human derived fibrin sealant applicator system.

ii. Needle applicator - a modification of the Vivostat™ spray applicator (automated applicator unit and spray applicator) (refer to Section 2.8.1). Unlike the spray applicator, no air is used to drive the application process and hence a slower delivery rate is achieved, with the intention of greater control and precision during the application process.

Aims of this study

To compare:

i. manually (bench) and automated processor (device) prepared novel fibrin sealant with regard to PSA prevention.
ii. the efficiencies of spray, needle and UNES gun application systems for prevention of PSAs.

6.2 Materials and Methods

This study utilised a rabbit uterine horn abrasion model (refer to Chapter 2), with animals

randomised to one of five experimental groups (Table 6.1). Volume of novel fibrin sealant applied to each experimental site was not dictated by the study protocol and was left to the discretion of the surgeon (Table 6.1). Spray and needle applications used the Vivostat™ 300µl applicator.

Treatment	Application Method (Vivostat™)	Mean Volume of Sealant Applied (ml)	Mean Fibrin I Conc^{n} (mg/ml)	Number of Animals	Number of Experimental Sites
Control	N/A	N/A	N/A	7	13
Novel Fibrin Sealant - Device Prepared	Spray	0.64±0.05	14.81±0.50	6	12
Novel Fibrin Sealant - Device Prepared	UNES Gun	1.05±0.09	15.66±1.30	6	12
Novel Fibrin Sealant - Device Prepared	Needle	0.88±0.05	16.87±0.79	6	12
Novel Fibrin Sealant - Bench Prepared	UNES Gun	0.80±0.09	20.10±2.81	6	12

Table 6.1: Summary of experimental group details.
Mean volumes and concentrations of fibrin sealant applied together with SEMs.

Surgical, termination (14 days post surgery) and histological procedures were as previously described (Chapter 2). Tissues were all processed to paraffin wax, sections cut at 5µm and stained with haematoxylin and eosin, with analysis provided by Cavalieri's Principal for determining absolute volumes (refer to Chapter 2).

6.3 Results

During this study there were no mortalities and no animal demonstrated morbidity associated with the experimental procedure.

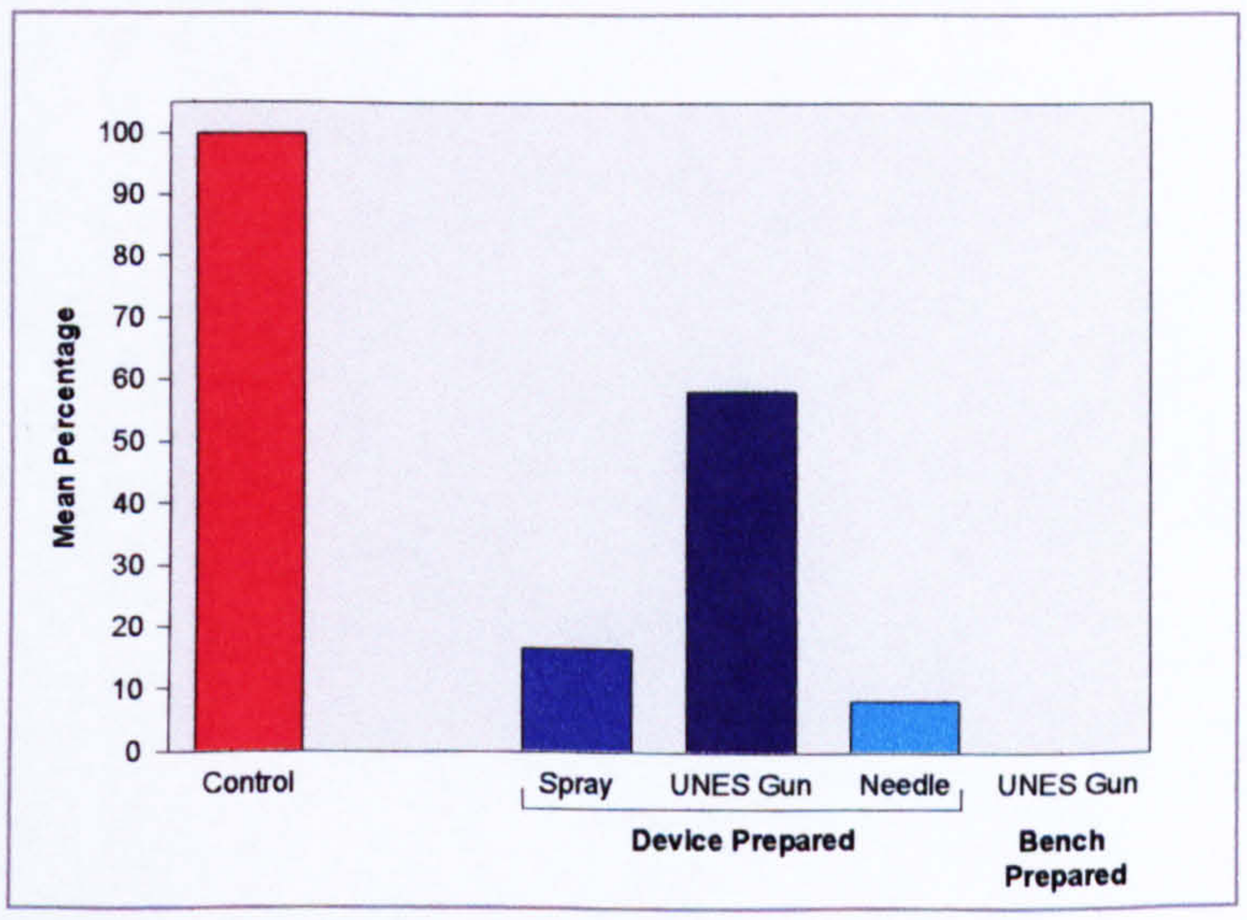
6.3.1 Macroscopic Results

All experimental animals demonstrated the formation of general PSAs associated with a laparotomy procedure, that is, PSAs between the peritoneal suture line and underlying tissues, for example, the fat pad over the bladder or the bladder itself (Appendix VI Table 6.1).

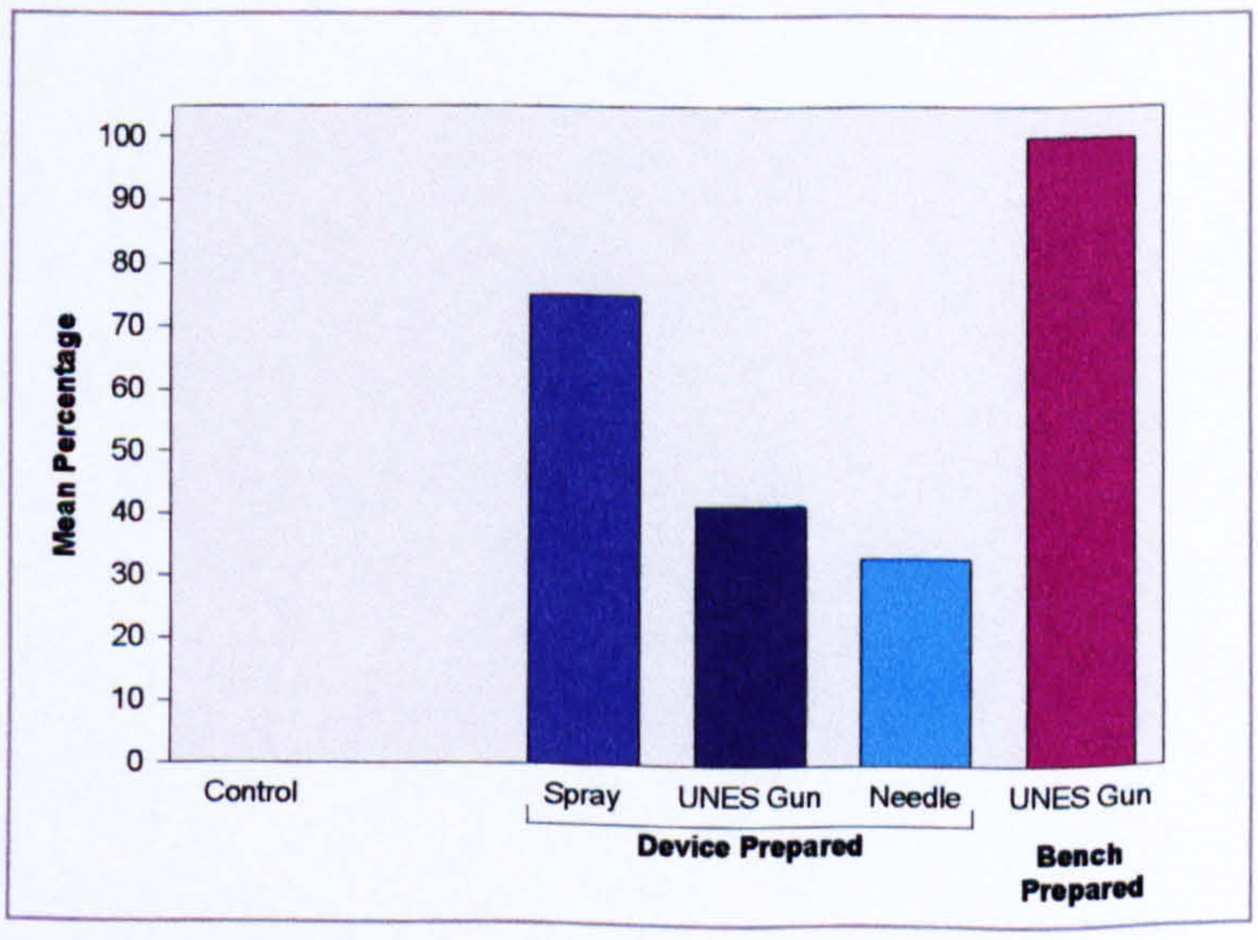
All control cases had PSAs present at all experimental sites, with all treatment groups

showing a reduction in experimentally-induced PSAs. Bench prepared (BP) UNES gun demonstrated a complete reduction of PSAs with an incidence of 0% PSA, device prepared (DP) needle 8.3%, DP spray 16.7% and DP UNES gun 58.3% incidence of PSAs at experimental sites (Graph 6.1, Appendix VI Table 6.1 and 6.2).

All treated groups demonstrated novel fibrin sealant remaining at experimental sites at 14 days post surgery, with incidence varying with the application method employed. DP needle showed the lowest incidence of 33.3%, followed by DP UNES gun with 41.7%, DP spray with 75% and finally the BP UNES gun with 100% incidence (Graph 6.2, Appendix VI Table 6.1 and 6.2).



Graph 6.1: Mean macroscopic PSA incidence.



Graph 6.2: Mean macroscopic novel fibrin sealant remaining incidence.

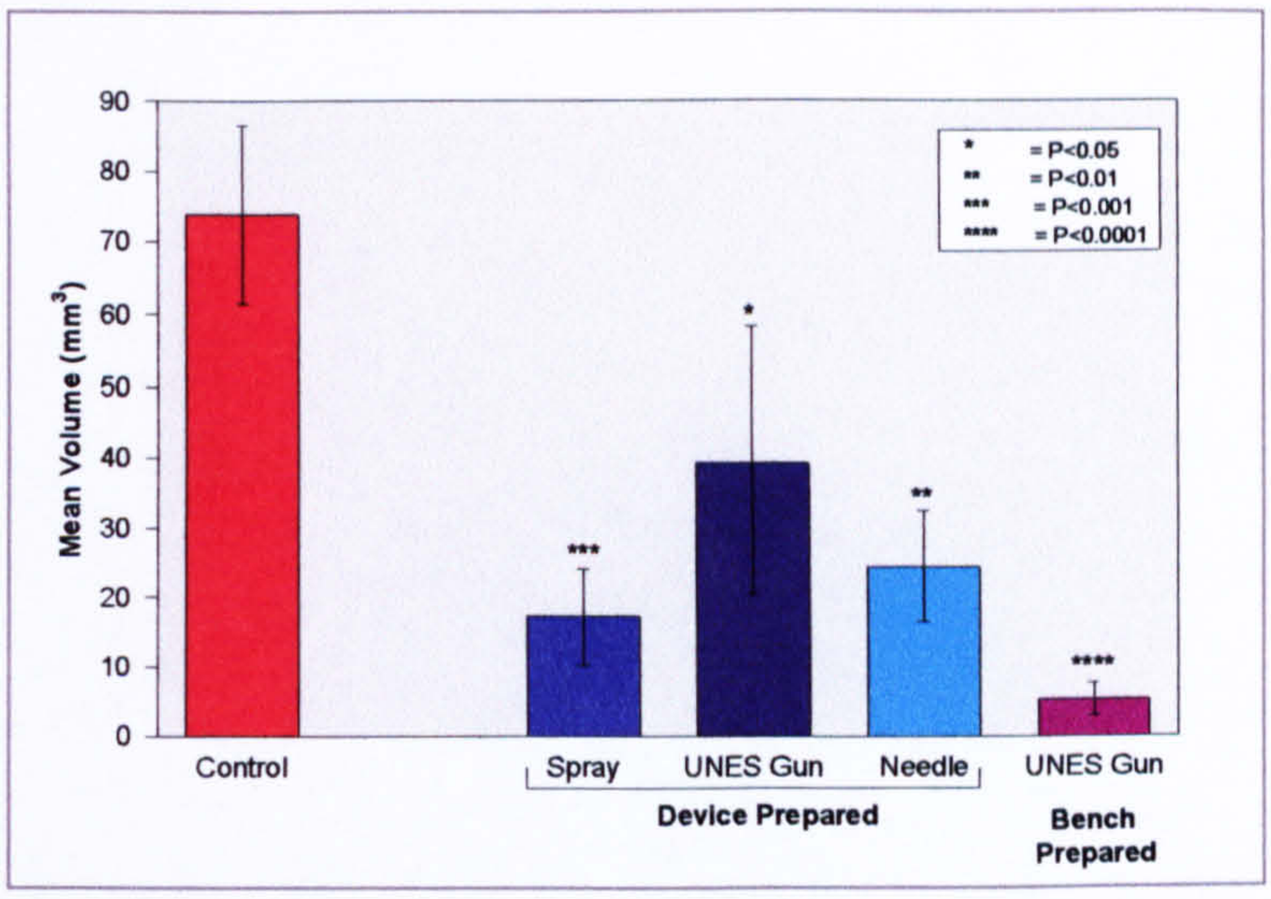
6.3.2 Microscopical Quantitative Results

Mean volume of PSAs for the control group was 73.93mm^3, DP spray application 17.32mm^3, DP UNES gun 39.37mm^3 and DP needle 24.66mm^3, with BP UNES gun 5.40mm^3 (Graph 6.3, Appendix VI Table 6.3). All treatment groups demonstrated significantly lower ($P<0.05$) mean volumes compared to controls, with no significant difference ($P>0.05$) between treatment groups (Appendix VI Table 6.4).

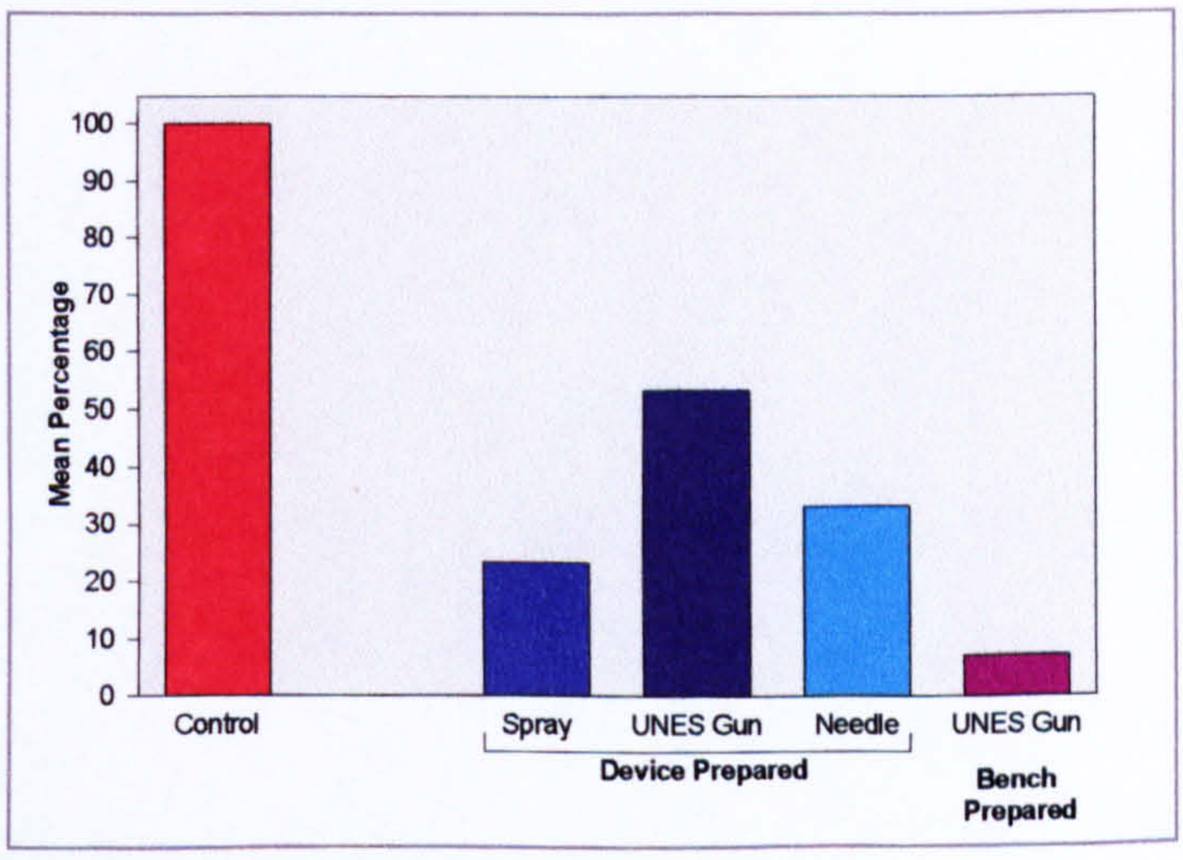
Assuming that controls have 100% volume of PSAs, BP UNES gun showed the greatest reduction with 7.31% volume, followed by DP spray with 23.43%, DP needle with 33.36% and finally DP UNES gun with 53.25% (Graph 6.4, Appendix VI Table 6.3).

Peritoneal tissue generation (PTG) mean volume for controls was 48.71mm^3, with DP spray 101.93mm^3, DP UNES gun 101.27mm^3, DP needle 96.31mm^3 and BP UNES gun 80.46mm^3 (Graph 6.5, Appendix VI Table 6.3). DP treatment groups all had significantly greater mean volumes ($P<0.01$) compared to controls whereas BP UNES gun showed a trend to have greater mean volume but not significantly ($P>0.05$) (Appendix VI Table 6.4). However there was no significant difference ($P>0.05$) between treatment groups (Appendix VI Table 6.5).

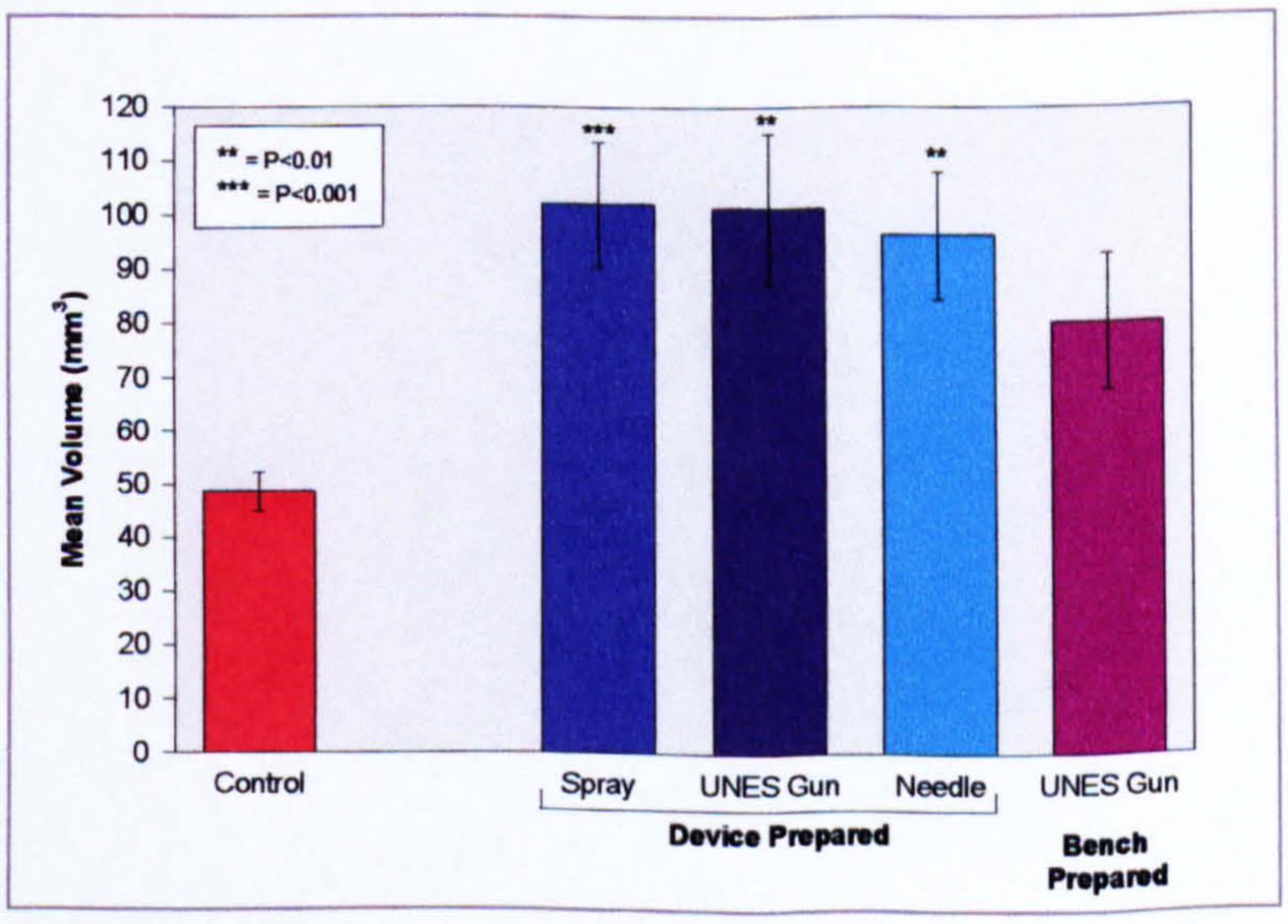
Mean volume of uterine horn tissue generation (UTG) showed a trend to be greater in treated groups, with controls having 12.42mm^3, DP spray 22.38mm^3, DP UNES gun 22.12mm^3, DP needle 17.17mm^3 and BP UNES gun 24.84mm^3 (Graph 6.6, Appendix VI Table 6.3). Only DP UNES gun showed a significant increase ($P>0.05$) compared to controls (Appendix VI Table 6.4), however there was no significant difference ($P>0.05$) between treated groups (Appendix VI Table 6.5).



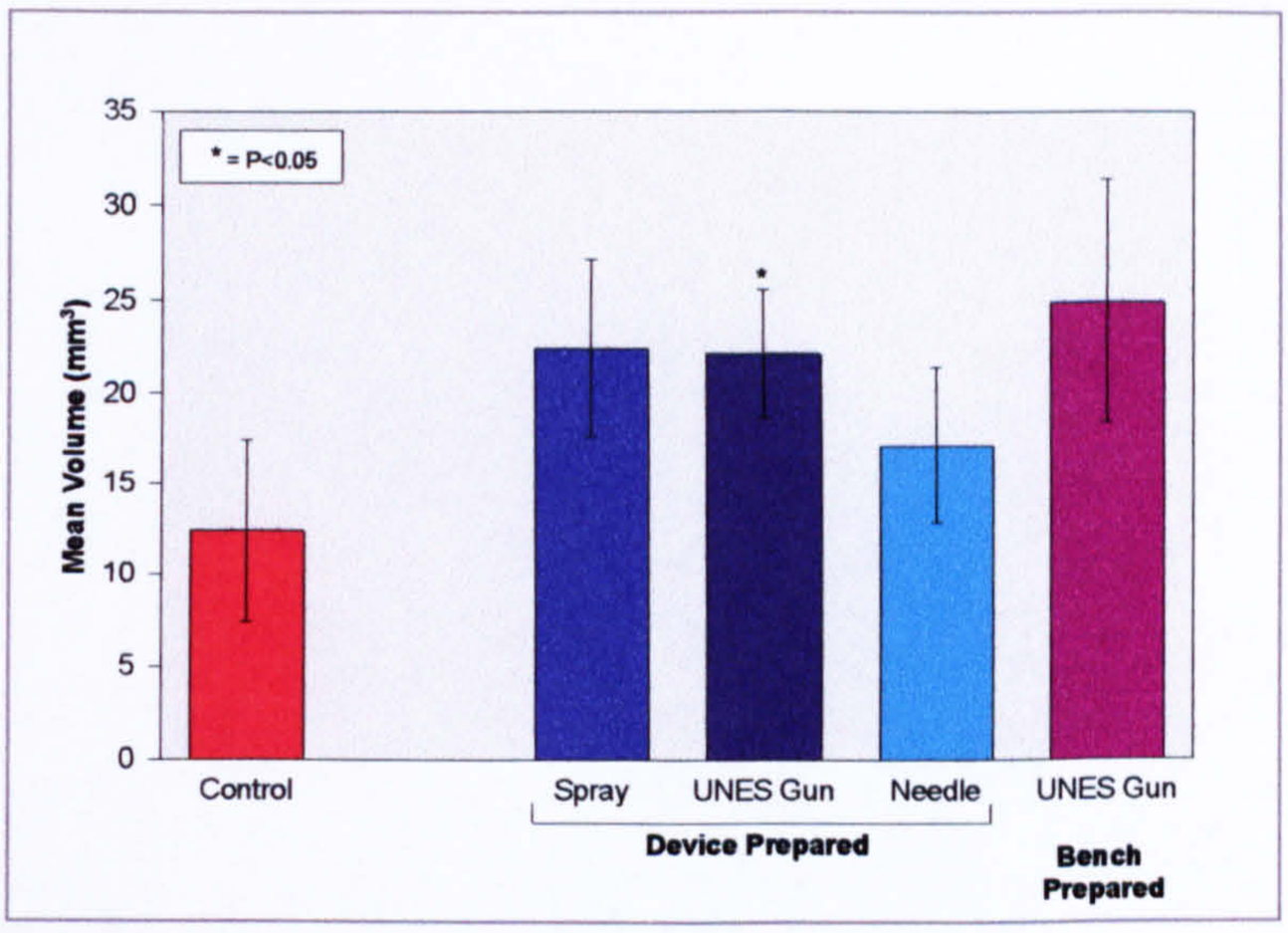
Graph 6.3: Mean PSA volumes
together with SEMs and statistical differences.



Graph 6.4: Mean PSA percentage volumes.



Graph 6.5: Mean PTG volumes
together with SEMs and statistical differences.



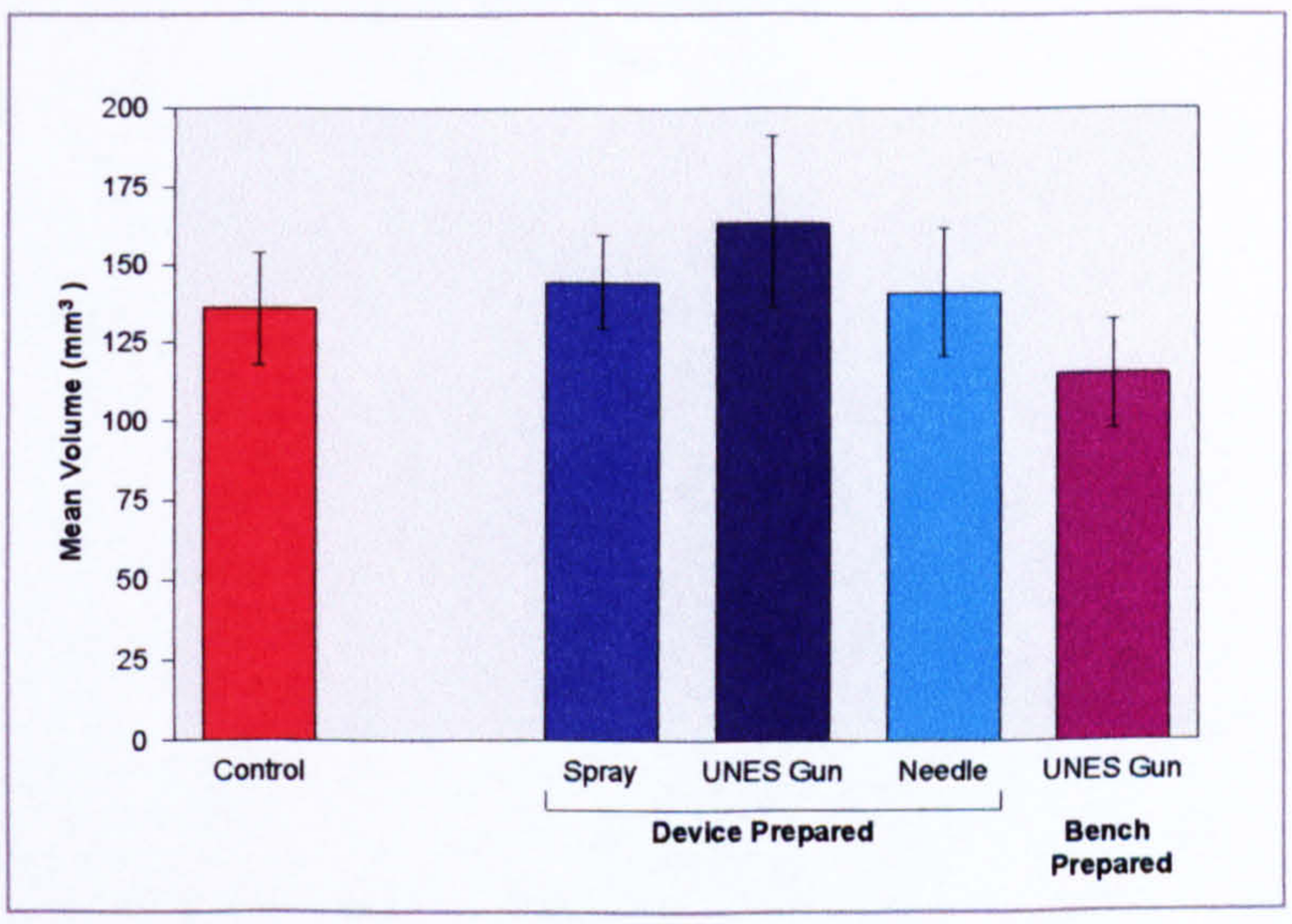
Graph 6.6: Mean UTG volumes
together with SEMs and statistical differences.

No significant differences ($P<0.05$) were seen between control and treated groups or within the treated groups with regard to mean volume of total tissue generated (TTG) (Graph 6.7, Appendix VI Tables 6.3, 6.4 and 6.5). The control group showed a mean volume of 136.07mm^3, DP spray 144.86mm^3, DP UNES gun 164.31mm^3, DP needle 141.99mm^3 and BP UNES gun 115.88mm^3.

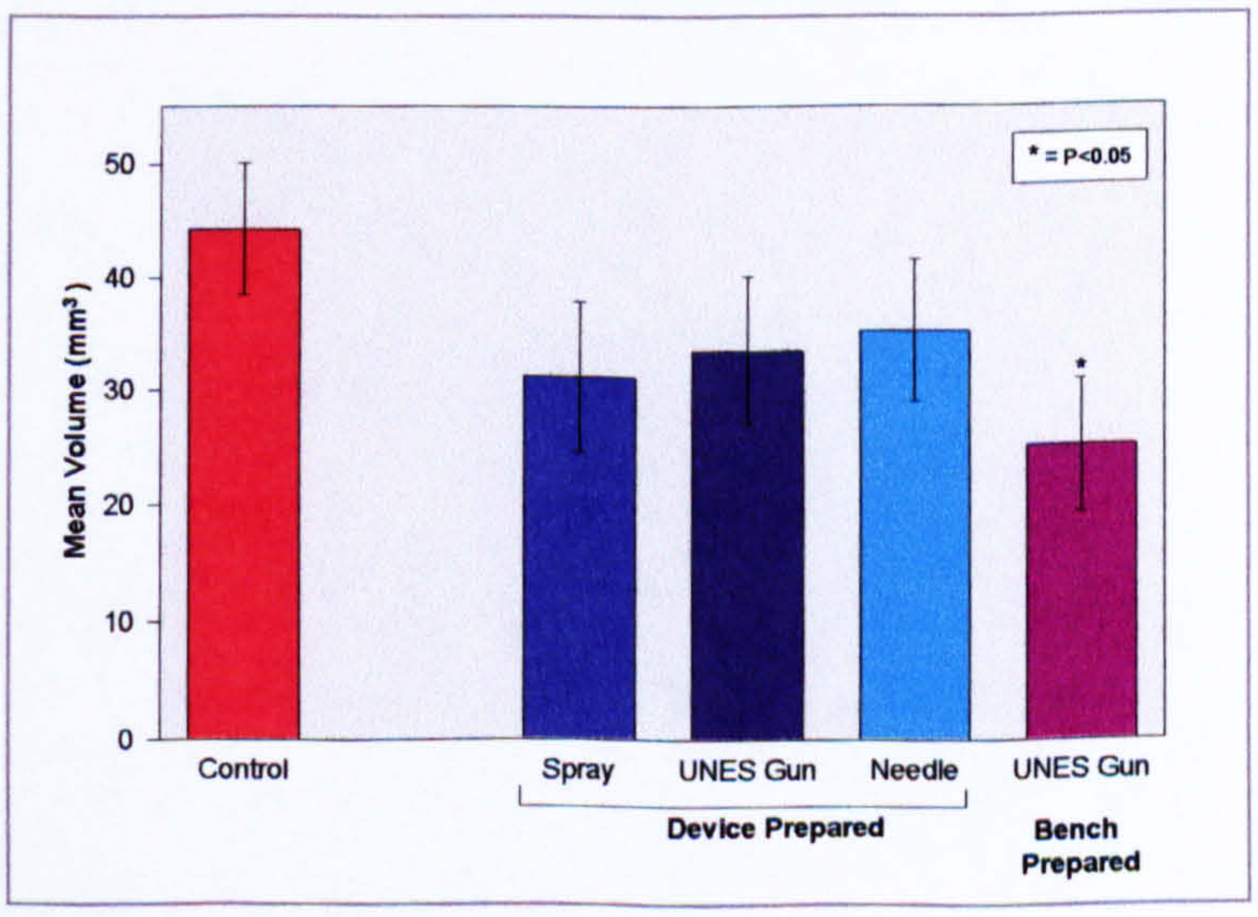
The control group demonstrated a mean volume of peritoneal damage of 44.29mm^3, with DP spray 31.32mm^3, DP UNES gun 33.73mm^3, DP needle 35.46mm^3 and BP UNES gun 25.32mm^3 (Graph 6.8, Appendix VI Table 6.3). Only the BP UNES gun group demonstrated a significantly lower ($P=0.024$) mean volume compared to controls (Appendix VI Table 6.4) with no significant differences ($P>0.05$) between treated groups (Appendix VI Table 6.5).

Mean volume of uterine horn damage was 20.26mm^3 for controls, 7.49mm^3 for DP spray, 12.87mm^3 for DP UNES gun, 11.93mm^3 for DP needle and 11.72mm^3 for BP UNES gun (Graph 6.9, Appendix VI Table 6.3). Only the DP spray group had a mean volume significantly lower ($P=0.0208$) than the control group (Appendix VI Table 6.4) and again, there was no significant difference ($P>0.05$) between the treatment groups (Appendix VI Table 6.5).

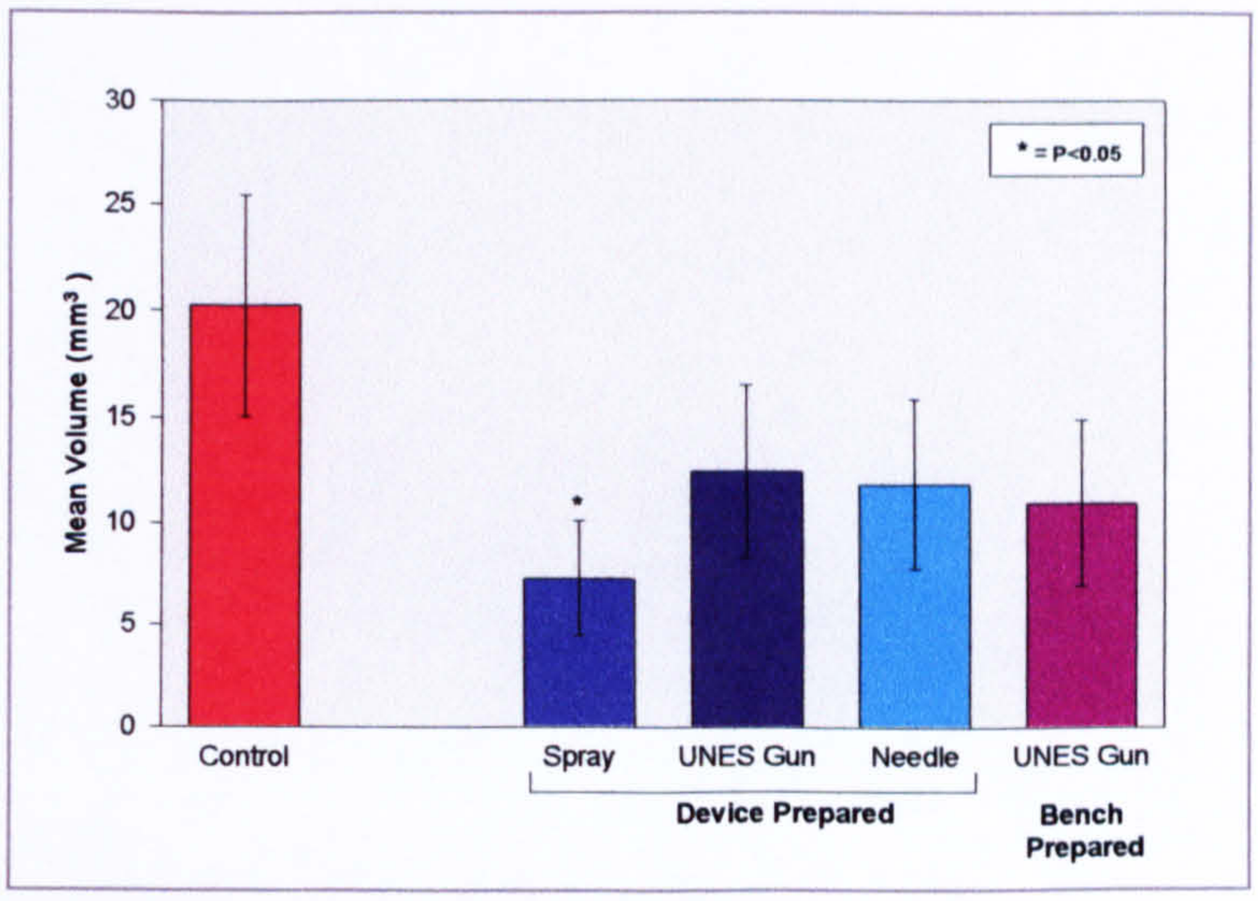
No significant differences (P>0.05) were seen for mean volume of peritoneal reaction, either comparing control and treated groups or between treatment groups (Appendix VI Tables 6.4 and 6.5). Mean values were 108.62mm^3 for controls, 109.62mm^3 for DP spray, 118.49mm^3 for DP UNES gun, 91.78mm^3 for DP needle and 88.64mm^3 for BP UNES gun (Graph 6.10, Appendix VI Table 6.3).



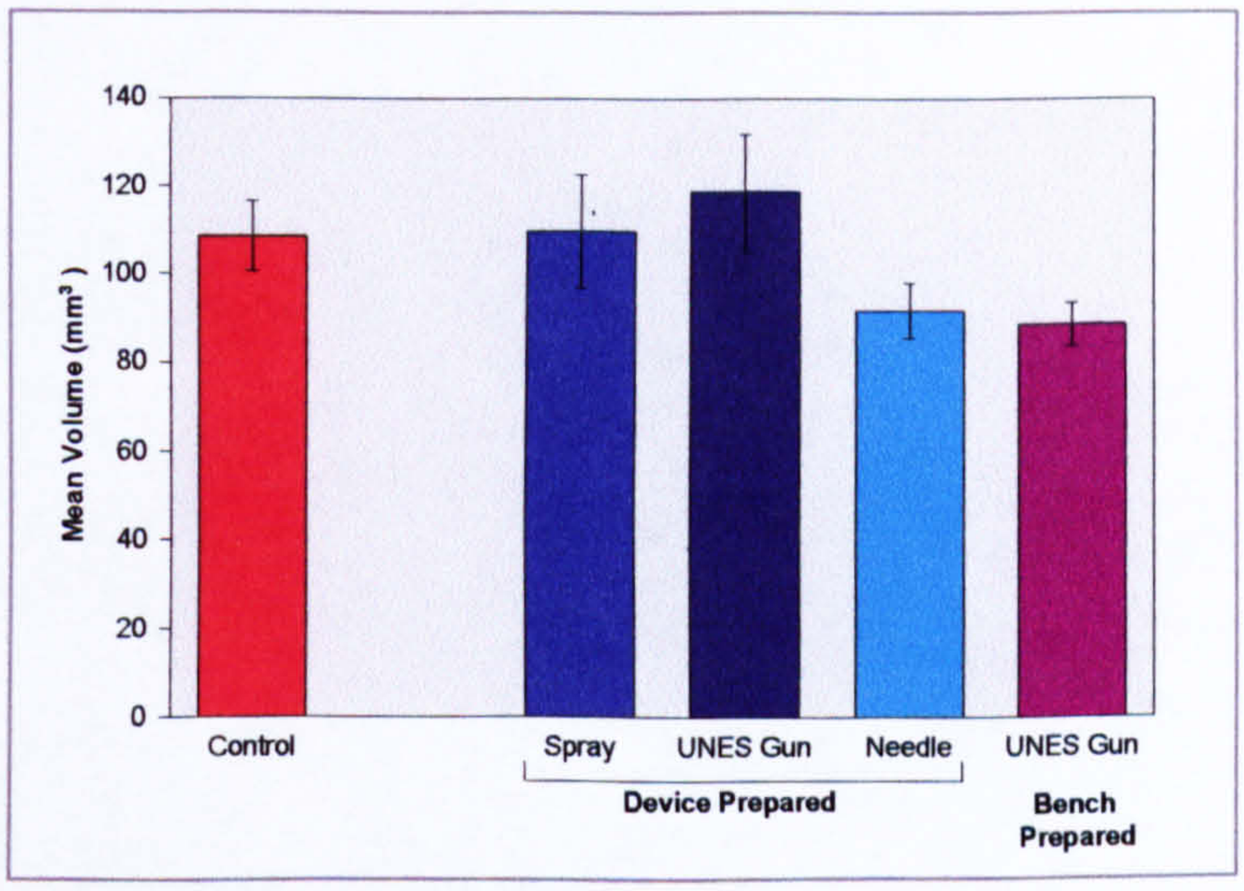
Graph 6.7: Mean TTG volumes
together with the SEMs and statistical differences.



Graph 6.8: Mean peritoneal damage volumes
together with the SEMs and statistical differences.



Graph 6.9: Mean uterine horn damage volumes
together with SEMs and statistical differences.

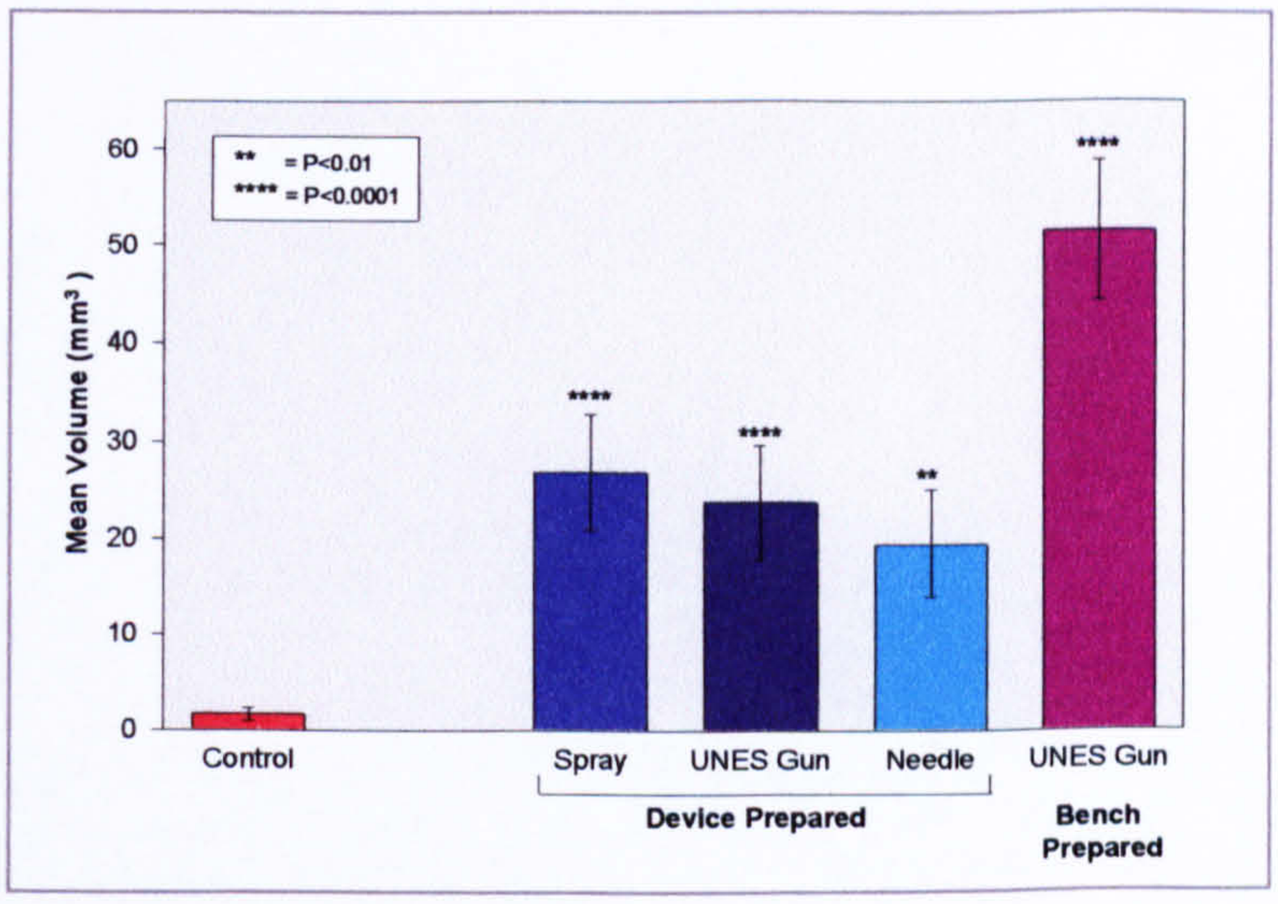


Graph 6.10: Mean peritoneal reaction volumes
together with the SEMs and statistical differences.

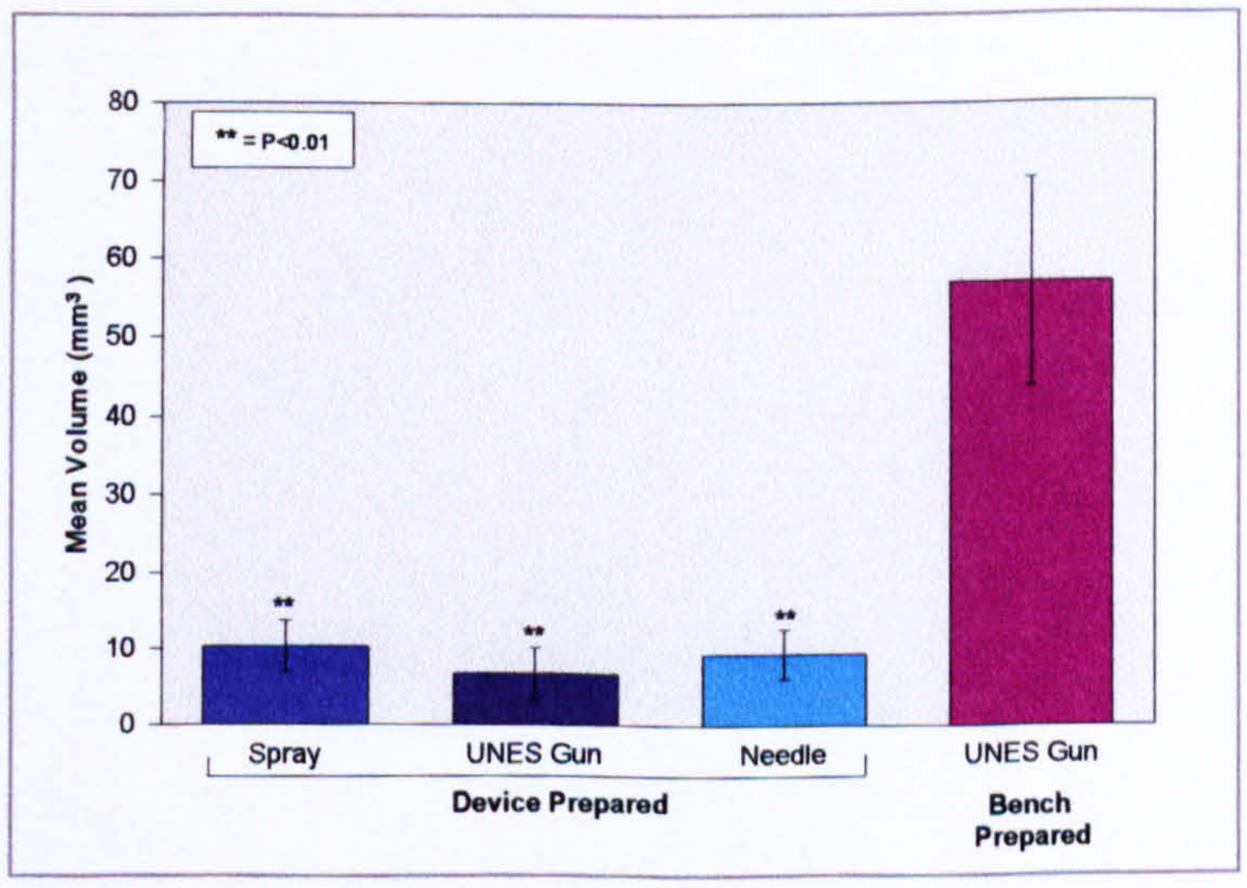
Mean volumes of cell concentration were significantly greater ($P<0.01$) for treated compared to control groups (Graph 6.11, Appendix VI Table 6.4). Controls showed a mean volume of 1.61mm^3, with DP spray 26.87mm^3, DP UNES gun 23.80mm^3, DP needle 19.63mm^3, and BP UNES gun 51.67mm^3 (Appendix VI Table 6.3). There was also a significant difference ($P=0.0028$) between device and bench prepared fibrin sealant groups (Appendix VI Table 6.5).

Novel fibrin sealant remaining at the completion of this 14 day study varied between device and bench prepared treatment groups, with bench prepared having significantly

greater (P=0.0014) volumes compared to device prepared (Graph 6.12, Appendix VI Table 6.5). Volume of sealant remaining was 10.43mm^3 for DP spray, 6.87 mm^3 for DP UNES gun, 9.40mm^3 for DP needle and 56.93mm^3 for BP UNES gun.



Graph 6.11: Mean cell concentration volumes together with SEMs and statistical differences.



Graph 6.12: Mean novel fibrin sealant remaining volumes together with SEMs and statistical differences.

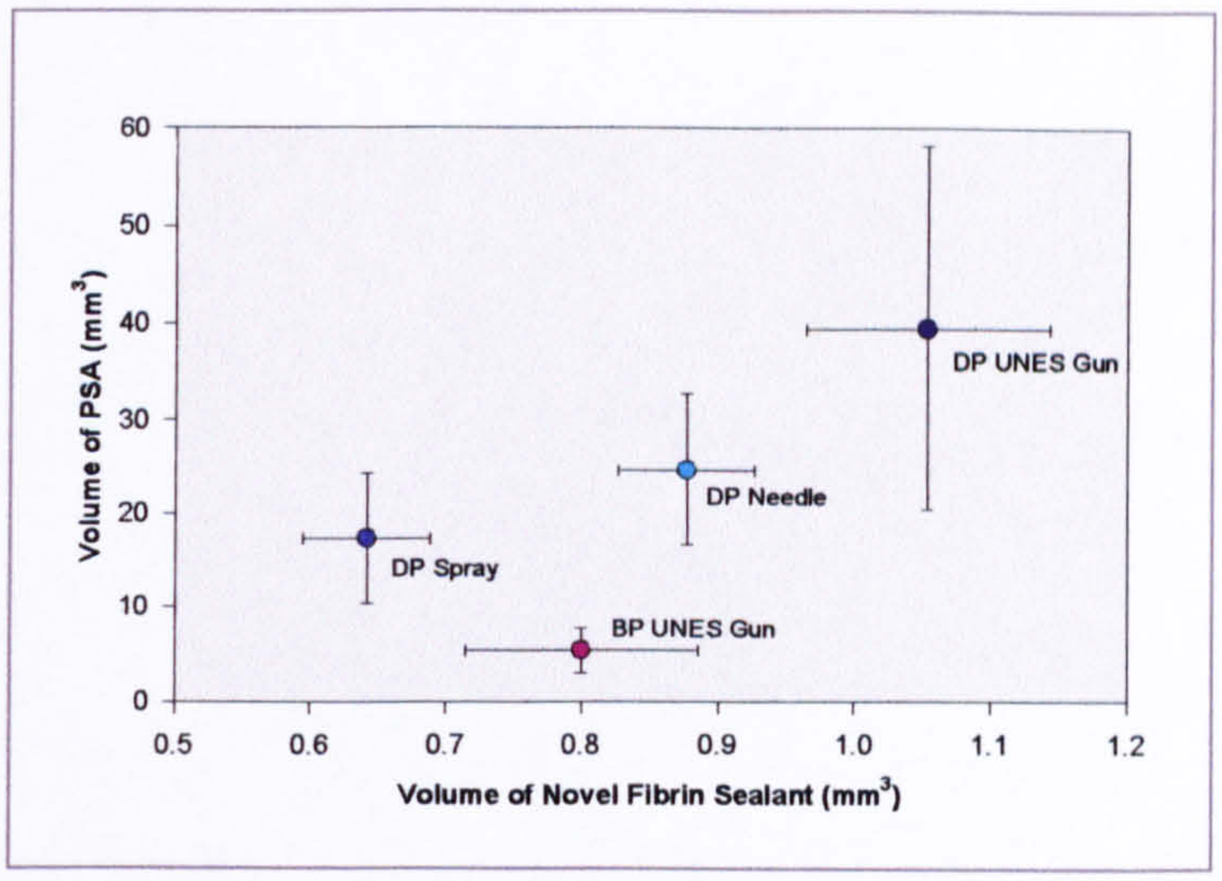
6.4 Discussion

Novel fibrin sealant, either device or bench prepared, applied using spray, needle or UNES gun systems significantly (P<0.05) reduced PSA formation, both incidence and volume, in the rabbit uterine horn abrasion model. This supports data from all previous studies investigating PSAs prevention with novel fibrin sealant.

This study demonstrated variation in incidence and mean volume of PSAs, although not statistically significant (P>0.05), between bench and device prepared novel fibrin sealant. Bench prepared sealant showed greater effectiveness in reducing both incidence and volume of PSAs compared to device prepared sealant. There were also differences, again not significant (P>0.05), between the device prepared fibrin sealant with regard to application methods employed, with spray producing the greater PSA reduction, followed by needle and then UNES gun.

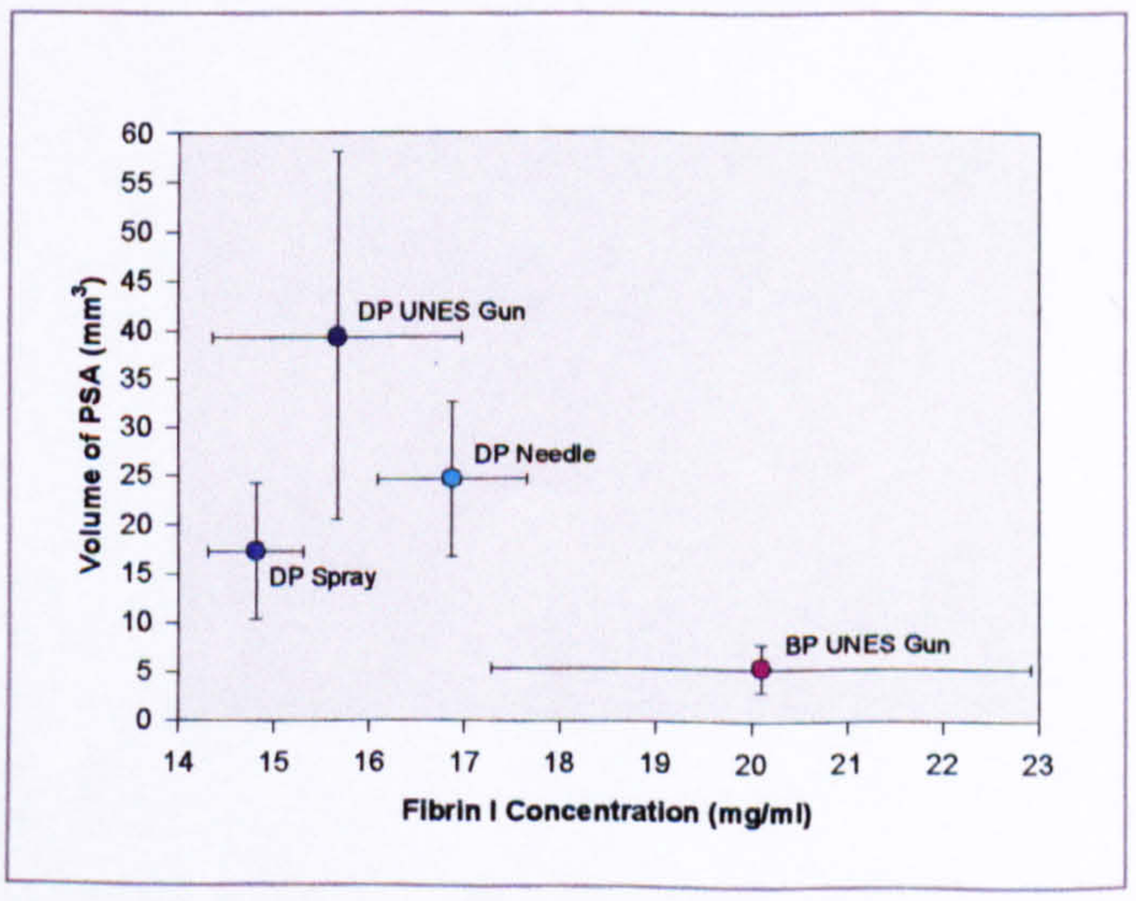
There are several factors which could be responsible for these variations: (i) volume of fibrin sealant applied; (ii) concentration of fibrin I in the fibrin sealant; (iii) cross-linking of the fibrin sealant; (iv) content of the fibrin sealant; and (v) coverage with the fibrin sealant. These will be discussed in turn.

i. Volume of fibrin sealant applied to experimental sites was determined by the surgeon during this study and hence varied between application methods. There were significant differences between the volume of sealant applied for several application methods (Appendix VI Table 6.6): mean volumes of DP spray were significantly lower (P<0.01) than DP UNES gun and DP needle; mean volumes of DP UNES gun were significantly greater (P<0.05) than DP needle. These variations appear to exert some effect on PSA prevention (Graph 6.13) with a trend for lower volumes of fibrin sealant applied resulting in lower PSA volumes. However no significant differences (P>0.05) were seen between treatment groups with regard to PSA volume. Hence volumes of fibrin sealant applied in this study appear to have little influence on PSA volume.



Graph 6.13: Mean PSA volume against mean volume of novel fibrin sealant applied together with SEMs.

ii. The concentration of fibrin I present prior to polymerisation to fibrin (II) sealant could also potentially affect PSA prevention ability. Previous studies have demonstrated that the minimum effective concentration of fibrin I in novel fibrin sealant for effective haemostasis is between 5 and 10mg/ml. All experimental groups used fibrin sealants which were well above this minimum value, with no significant differences ($P>0.05$) demonstrated between treatment groups (Appendix VI Table 6.6). In addition there was no correlation between concentration of fibrin I and PSA volume (Graph 6.14). Hence concentrations of fibrin I in this study do not appear to be responsible for the differences seen in PSA reduction ability.



Graph 6.14: Mean PSA volume against fibrin I concentration together with SEMs.

iii. Variation in the degree and effectiveness of cross-linking and polymerisation of fibrin I to fibrin II in the fibrin sealant is another potentially variable factor when using automated and non-automated systems, with automated techniques designed to optimise mixing and hence polymerisation. Although the non-automated UNES gun was the least effective of the DP methods, BP UNES gun was the most effective technique of the study as a whole. This, in addition to no significant differences between automated and non-automated application methods, appears to eliminate this as a cause of the different efficacies seen.

iv. Analysis of bench and device prepared human derived fibrin sealants revealed no differences in protein content with regard to fibrin I, biotin-batroxobin and avidin-agarose. However significantly ($P<0.01$) greater volumes were present at 14 days for BP sealant compared to DP sealant. No further investigations into differences between BP and DP sealants were carried out due to no significant difference ($P<0.05$) in PSA reduction.

v. Variations in intervention of PSAs seen in this study are most probably attributed to the way in which the sealant was applied to injured surfaces. In order to achieve complete PSA prevention, it is assumed that a continuous layer of fibrin sealant is needed to separate the surfaces. In these conditions fibrous exudate produced on each injured surface can not reach the apposed injury or other tissue sites, hence fibrinous adhesions are prevented. From this study, is appears that this is attained most regularly by UNES gun for bench prepared novel sealant, followed by, in order of effectiveness, spray, needle and then UNES gun for device prepared novel fibrin sealant.

This study indicates that control and treated cases generated similar volumes of tissue in response to abrasion injury but the location/type of tissue generated varied. Control cases demonstrated greatest volumes of PSA, followed by PTG, UTG and then free floating tissue generation (FFTG) (tissue generation which, in transverse section, does not appear to be attached to either the uterine horn or peritoneal wall), whereas treated cases

showed largest mean volumes of PTG, PSA or UTG and least in FFTG. Hence treatment with novel fibrin sealant prevents tissue generated from injury sites from forming PSAs but does not prevent tissue being generated in response in injury.

Treatment with novel fibrin sealant has a slight protective effect with regard to tissue damage seen at 14 days, with treated groups demonstrating a trend for lower mean damage volumes. However significant differences ($P<0.05$) were only seen for BP UNES gun for peritoneal damage and DP spray for uterine horn damage. From previous studies (refer to Chapter 3) it has been demonstrated that damage associated with the abrasion injury in the rabbit uterine horn abrasion model follows the normal progression for wound healing: damage from the initial scraping injury only is seen for the first few days, followed by an increase in mean volume associated with clearing of necrotic underlying and surrounding tissues, which in turn is followed by a decrease in damage volume as new wound replacement tissue is laid down. By 14 days the final replacement process is occurring with the difference seen between control and treated cases in this study most probably due to novel fibrin sealant having a protective effect on injured and surrounding tissues, limiting the volume of underlying and surrounding tissue necrosis and hence damage. The mechanisms involved in this tissue protection could either be due to the sealant providing a barrier between the injury site and peritoneal cavity and its contents, or the sealant having biochemical effects on tissue. Further analysis will hopefully clarify these mechanisms further.

Treatment with novel fibrin sealant had little to no effect on the volume of reaction seen in the peritoneum at 14 days post injury. This appears to support the above hypothesis that fibrin sealant protects injured tissue from damage due to a barrier effect as opposed to exerting a biochemical influence.

As observed in all previous novel fibrin sealant studies, treatment with novel fibrin sealant using this rabbit uterine horn abrasion model induced a local cell reaction, seen as areas of cell concentrations. This is most probably an immune response mounted to the introduction of a foreign protein (human blood derived fibrin). However even with this

cellular response, novel fibrin sealant significantly reduced the formation of PSAs. The significant difference ($P<0.01$) seen between device and bench prepared fibrin sealant with regard to cell concentration is most probably due to bench prepared sealant remaining *in vivo* longer than device prepared, with longer residency time leading to a more sustained reaction.

6.5 Conclusions

i. Novel fibrin sealant, either device or bench prepared, applied using spray, UNES gun or needle systems, significantly ($P<0.05$) reduced PSA formation in the rabbit uterine horn abrasion model.
ii. There were no significant differences ($P>0.05$) between mean volume of PSAs for device and bench prepared fibrin sealant or application techniques used, however the trend for PSA reduction was BP UNES gun (93%), DP spray (77%), DP needle (67%) and DP UNES gun (56%).
iii. Differences in reduction of PSAs seen with different preparation and application methods are most probably due to the coverage of sealant over injured surfaces.
iv. Prevention of PSAs by novel fibrin sealant appeared to be due to its acting primarily as a physical barrier.

7.0 Comparison of Vivostat™ System Human Derived Fibrin Sealant, Tissucol® and CRYO for the Prevention of PSAs in the Rabbit Uterine Horn Abrasion Model

7.1 Introduction

Much interest has been shown in the use of fibrin sealants as surgical tools for haemostasis, tissue sealing and reduction of PSAs (refer to Chapter 1). With regard to PSA reduction, the absolute value of the different fibrin sealants investigated and their benefits over others is difficult to assess since previous studies have used a variety of experimental models and qualitative analytical procedures. Studies investigating Vivostat™ system human derived fibrin sealant (novel fibrin sealant) (refer to Chapters 4 and 6) have demonstrated the significant reduction and near complete prevention of PSAs using a definitive experimental model together with strict quantitative analyses. No such comprehensive information is accessible on commercially available fibrin sealants.

Tissucol® (Immuno AG, Vienna, Austria) was the first commercially available fibrin sealant utilising pooled human fibrinogen concentrate. Like many subsequent sealants, Tissucol® uses a two component system:

i. pooled human fibrinogen (in an aprotinin solution); and
ii. bovine thrombin (in a calcium chloride solution),

with the mixing of these leading to the formation of fibrin II polymerised sealant. Many studies have investigated the efficiency of Tissucol® to reduce PSAs with most being supportive of its use (Lindenberg & Lauritsen, 1984; Lindenburg *et al.*, 1985; Larsson *et al.*, 1986; De Laco *et al.*, 1994).

Legislation against the use of pooled fibrinogen products introduced in the United States in 1978 prompted the search for preparation systems using autologous fibrinogen resulting in autologous fibrin sealants. The method of CRYO precipitation was established to obtain autologous fibrinogen from blood over coming the problems of

pooled blood products. "CRYO" fibrin sealants, like Tissucol® still require the addition of exogenous thrombin in order for the autologous fibrinogen to form fibrin I. Generally, studies detailing the use of CRYO fibrin sealants for prevention against PSA formation are supportive (CRYO: Sheppard *et al.*, 1993; Ethanol precipitate: Weiss-Fogh *et al.*, 1990), however the clinical use of CRYO sealants has been limited by its time consuming production.

Aims of this study

i. Assess the ability of:
    a. Vivostat™ system human derived fibrin sealant (novel fibrin sealant)
    b. CRYO at two different bovine thrombin concentrations (4 and 500 units)
    c. Tissucol® at two different bovine thrombin concentrations (4 and 500 units)

    to reduce/prevent PSAs compared to controls in a definitive rabbit uterine horn abrasion model using quantitative analysis
ii. Identify possible mechanisms in which these potential PSA preventative agents are working

7.2 Materials and Methods

A summary of experimental groups together with volumes of fibrin sealant applied are shown in Table 7.1.

Treatment Group	Number of		Mean Volume of Sealant Applied (ml)	Mean Fibrin I Conc^{n} (mg/ml)
	Animals	Experimental Sites		
Control	7	13	N/A	N/A
Novel Fibrin Sealant (Needle Application)	6	12	0.93±0.06	14.90± 2.41
Tissucol®	6	Total: 11		17.50-28.75
- 4 Units Bovine Thrombin		5	0.50±0.03	
- 500 Units Bovine Thrombin		6	1.12±0.11	
CRYO	6	Total: 12		10.64±
- 4 Units Bovine Thrombin		6	0.47±0.02	0.84
- 500 Units Bovine Thrombin		6	0.33±0.03	

Table 7.1: Experimental group details.
Number of animals and experimental sites and mean volume and concentration of fibrin

sealant applied together with SEMs.

A rabbit uterine horn abrasion model was utilised in this study with standard surgical, termination (14 days post surgery) and histological procedures carried out (refer to Chapter 2). Tissucol® and CRYO were prepared as the manufacturer's instructions (refer to Chapter 1 and Appendix XII).

For the application of novel fibrin sealant a 300µl Vivostat™ applicator was used, with Duploject systems (refer to Chapter 2) used for Tissucol® and CRYO (as recommended by suppliers).

Resected tissues were processed to paraffin wax, 5µm sections cut and stained with haematoxylin and eosin and Cavalieri's analysis was carried out for determination of absolute volumes (refer to Chapter 2).

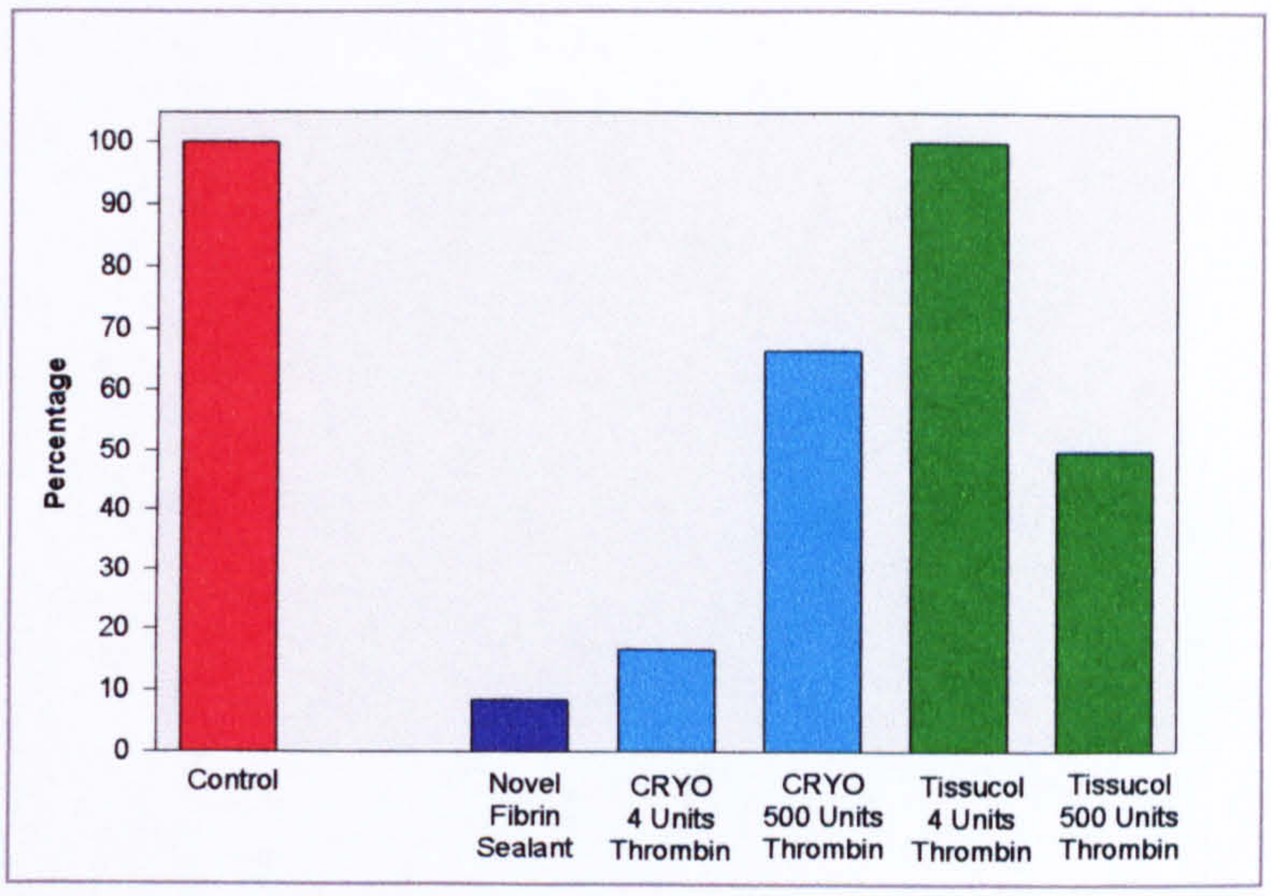
7.3 Results

There were no mortalities in this study and no animal demonstrated morbidity from the experimental procedure.

All animals demonstrated the formation of PSAs associated with laparotomy, that is, PSAs involving the peritoneal suture line and one or more of the following: omentum, fat pad over the bladder, or the bladder (Appendix VII Table 7.1).

7.3.1 Macroscopic Results

All of the control groups (13/13) showed PSAs joining the abraded areas of the peritoneal wall and uterine horn. The macroscopic incidence of experimentally induced PSAs for treatment groups was 8.3% for novel fibrin sealant, 16.7% CRYO 4 units thrombin, 66.7% CRYO 500 units thrombin, 100% Tissucol® 4 units thrombin and 50% Tissucol® 500 units thrombin (Graph 7.1; Appendix VII Table 7.1).

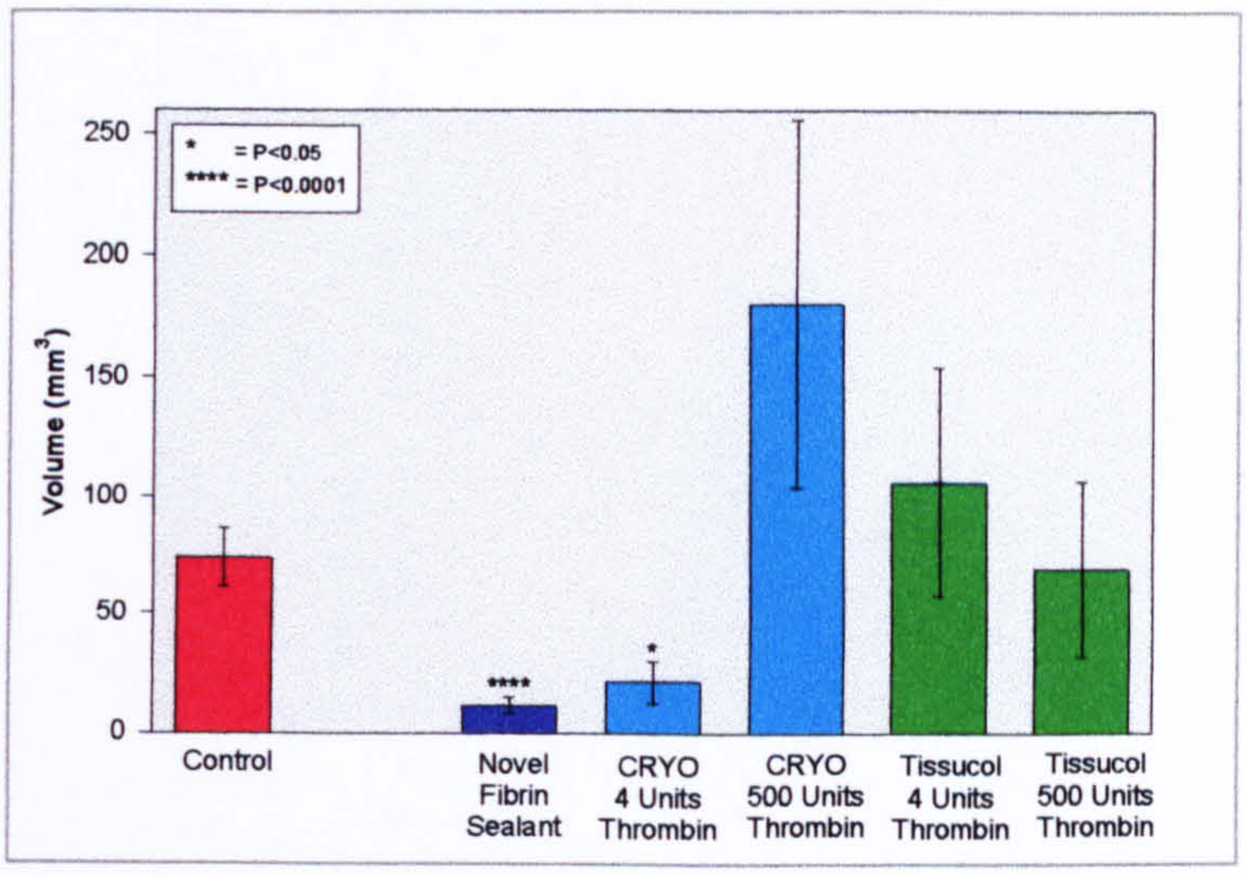


Graph 7.1: Macroscopic PSA incidence.

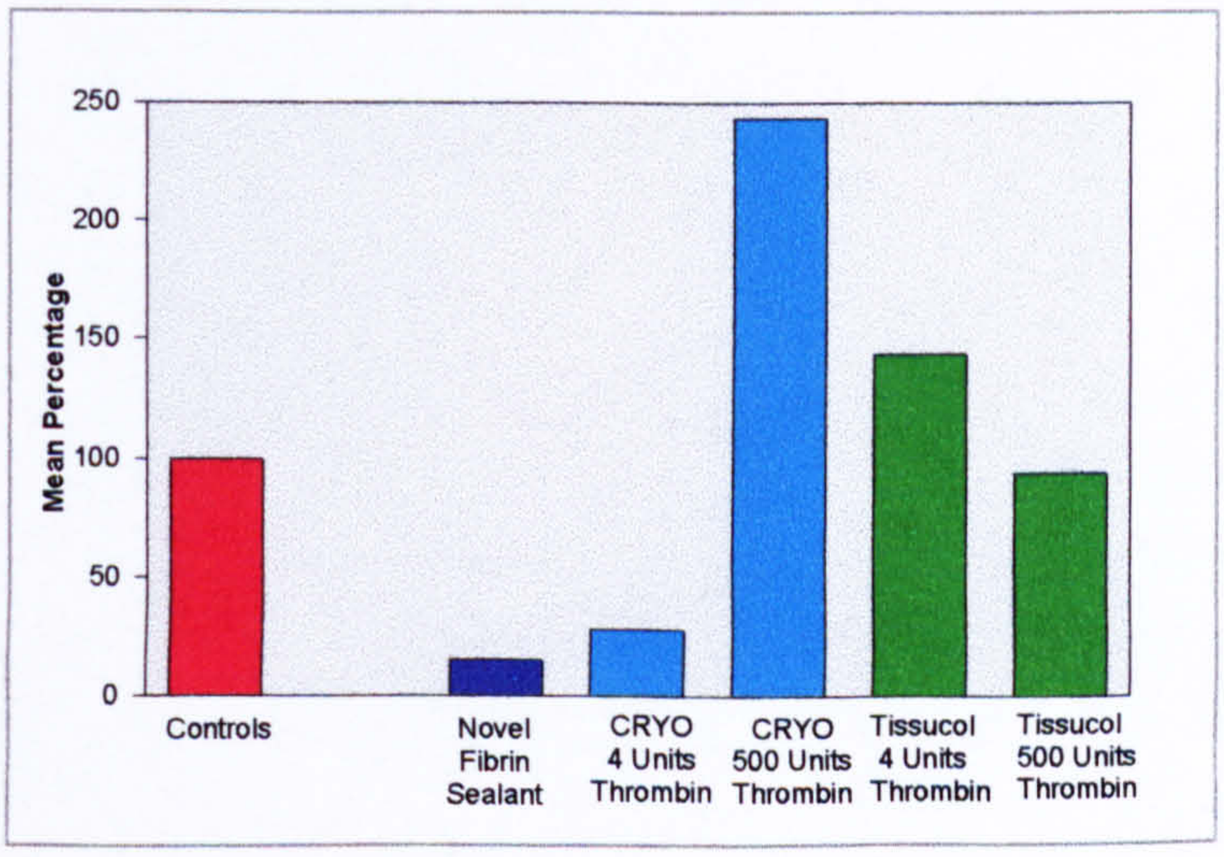
7.3.2 Microscopic Quantitative Results

Mean volumes of PSAs were 73.93mm^3 for the control group (Figure 7.1), 11.30mm^3 for the novel fibrin sealant treated group (Figure 7.2), 21.06 and 179.98mm^3 for the CRYO treated groups with 4 and 500 units thrombin respectively (Figures 7.3 and 7.4) and 106.12 and 69.42mm^3 for the Tissucol® treated groups with 4 and 500 units thrombin respectively (Figures 7.5 and 7.6) (Graph 7.2; Appendix VII Table 7.2). Significant reduction in mean PSA volume compared to controls was seen with novel fibrin sealant ($P<0.001$) and CRYO ($P=0.0152$) 4 units thrombin treated groups only (Appendix VII Table 7.3) .

Assuming that the control group demonstrated 100% PSA formation, the percentage volume of PSAs for each treatment was 15.28% novel fibrin sealant, 28.49% CRYO (4 units thrombin), 243.46% CRYO (500 units thrombin), 143.55% Tissucol® (4 units thrombin) and 93.90% Tissucol® (500 units thrombin) (Graph 7.3; Appendix VII Table 7.2).



Graph 7.2: Mean PSA volumes
together with SEMs and statistical differences.



Graph 7.3: Mean PSA percentage volume.

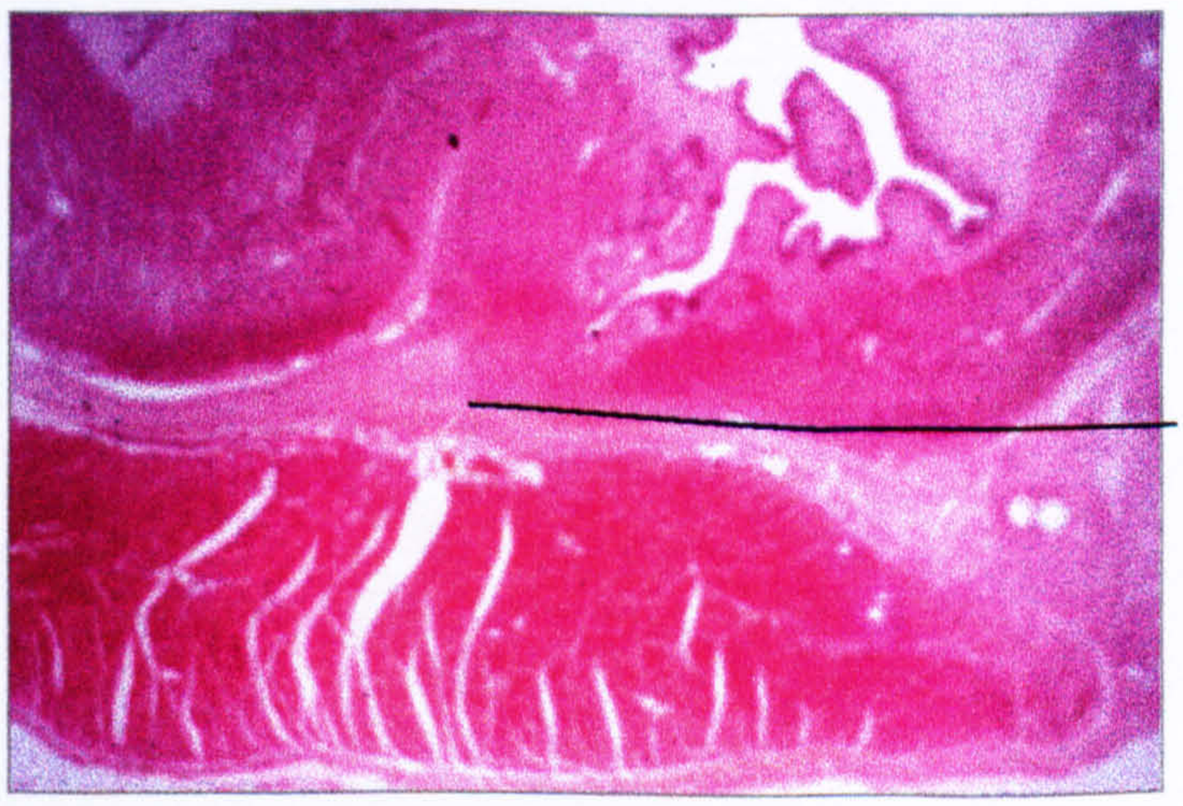


Figure 7.1: Control Case
(M_o x2)

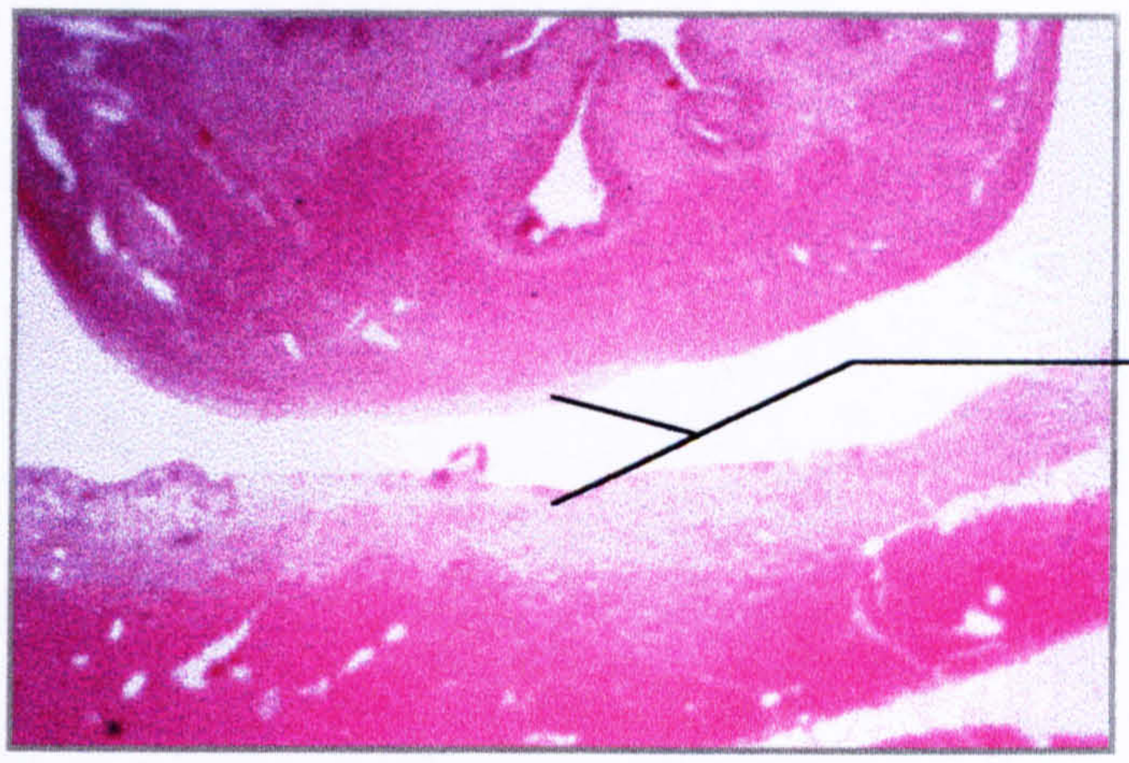


Figure 7.2: Novel fibrin sealant treated case.
(M_o x2)

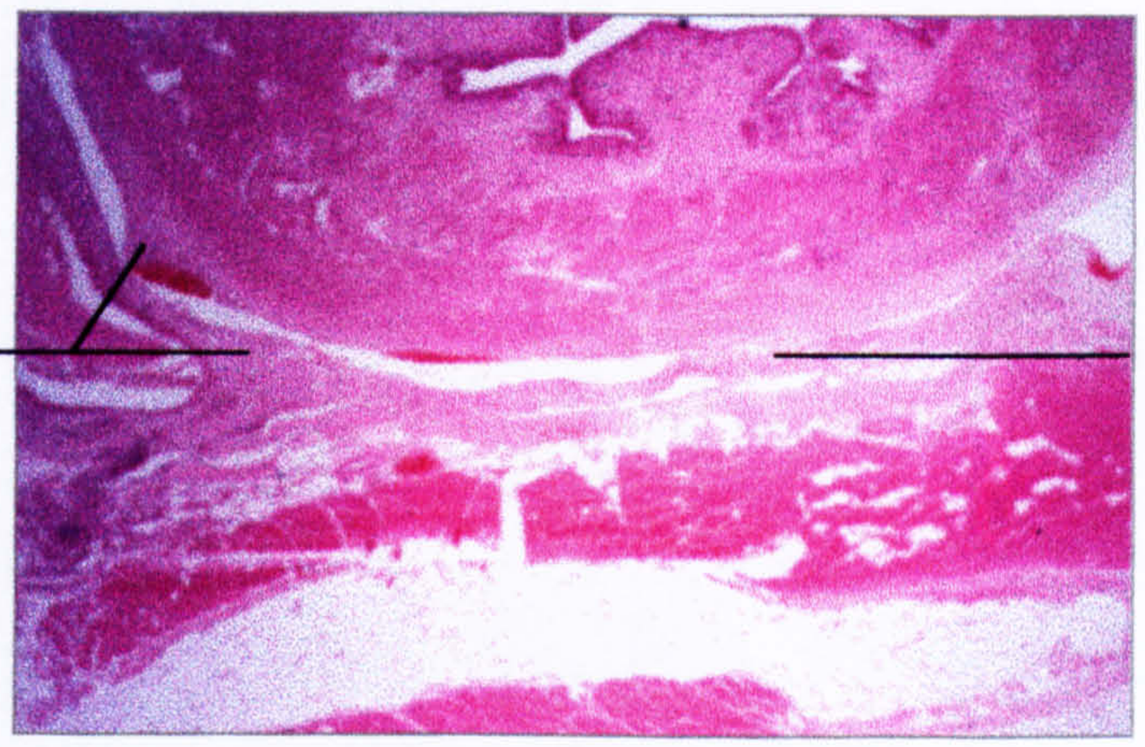


Figure 7.3: CRYO (4 units thrombin) treated case
(M_o x2)

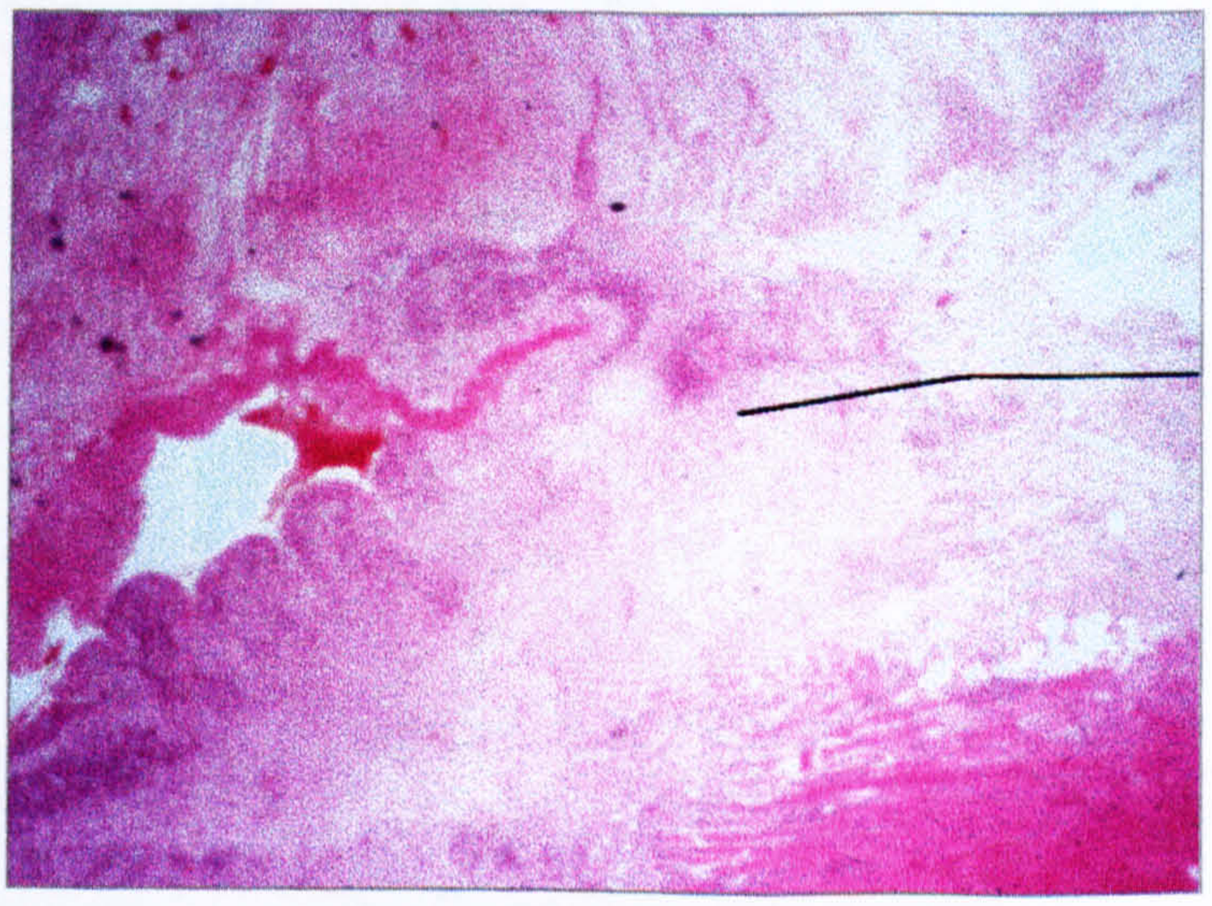


Figure 7.4: CRYO (500 units thrombin) treated case.
(M_o x2)

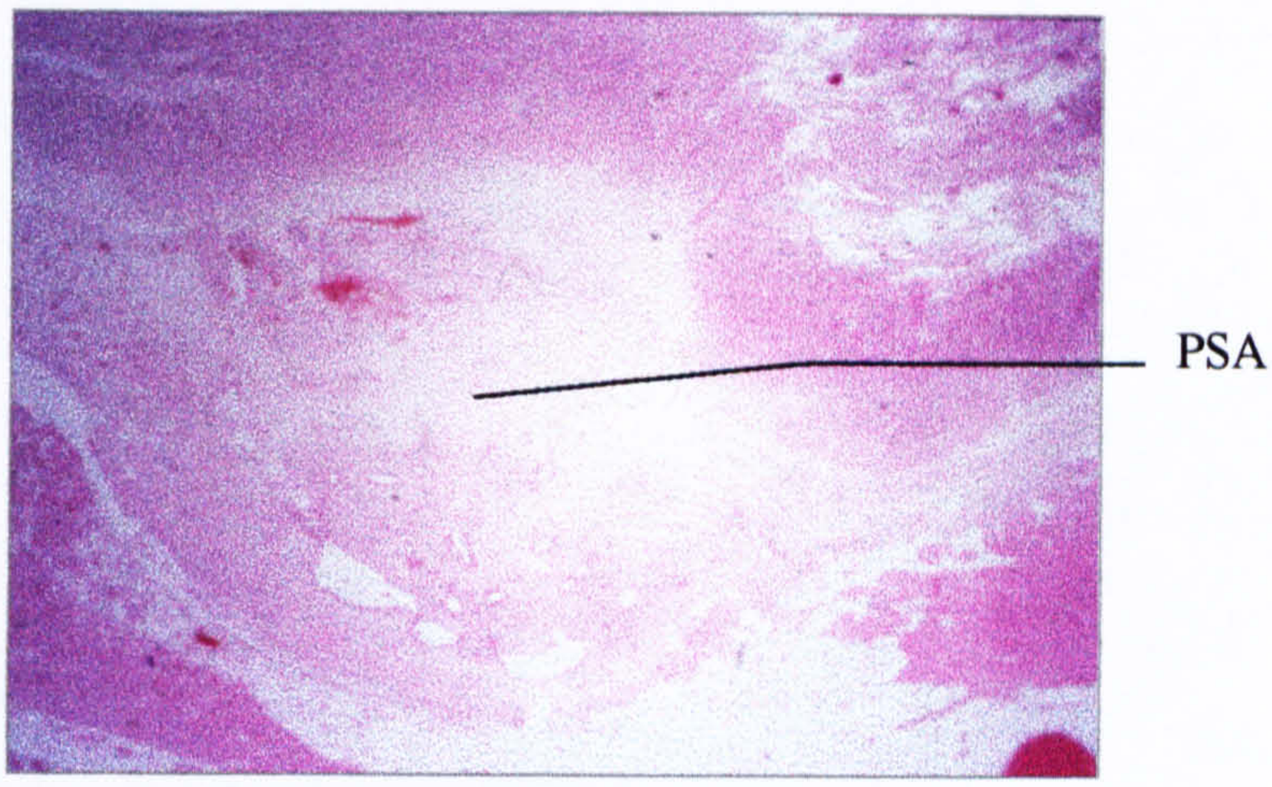


Figure 7.5: Tissucol (4 units thrombin) treated case.
(M_o x2)

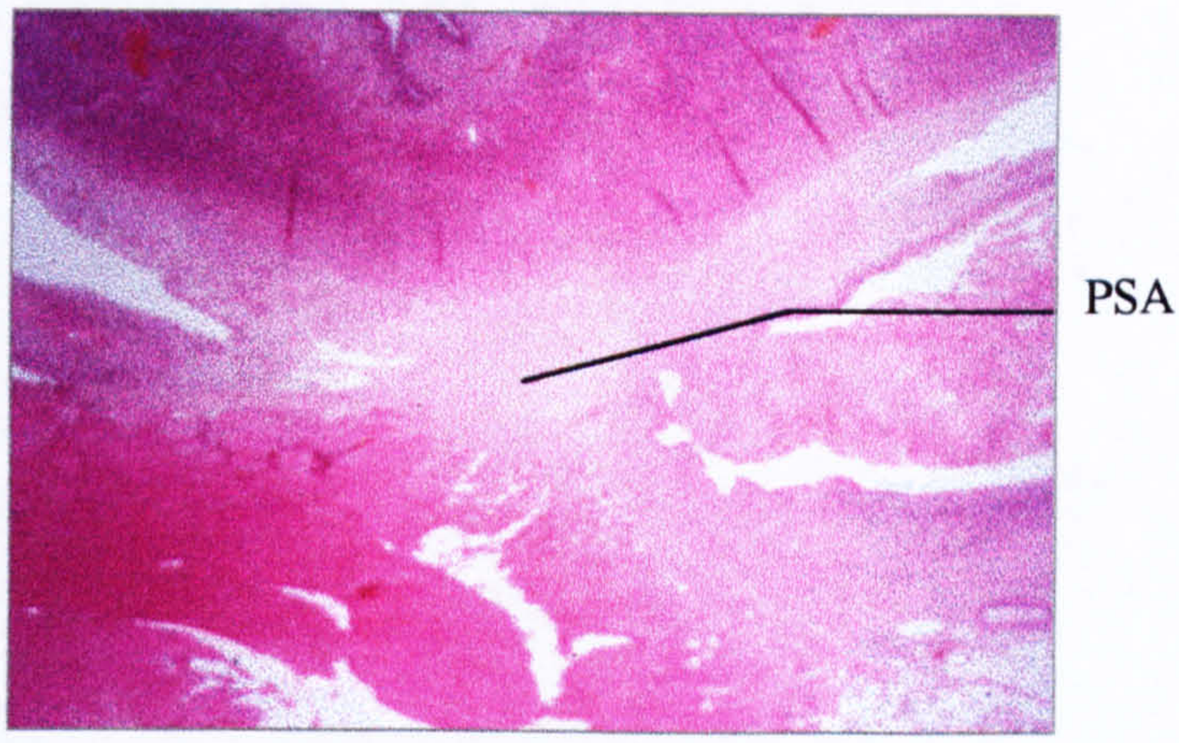


Figure 7.6: Tissucol (500 units thrombin) treated case.
(M_o x2)

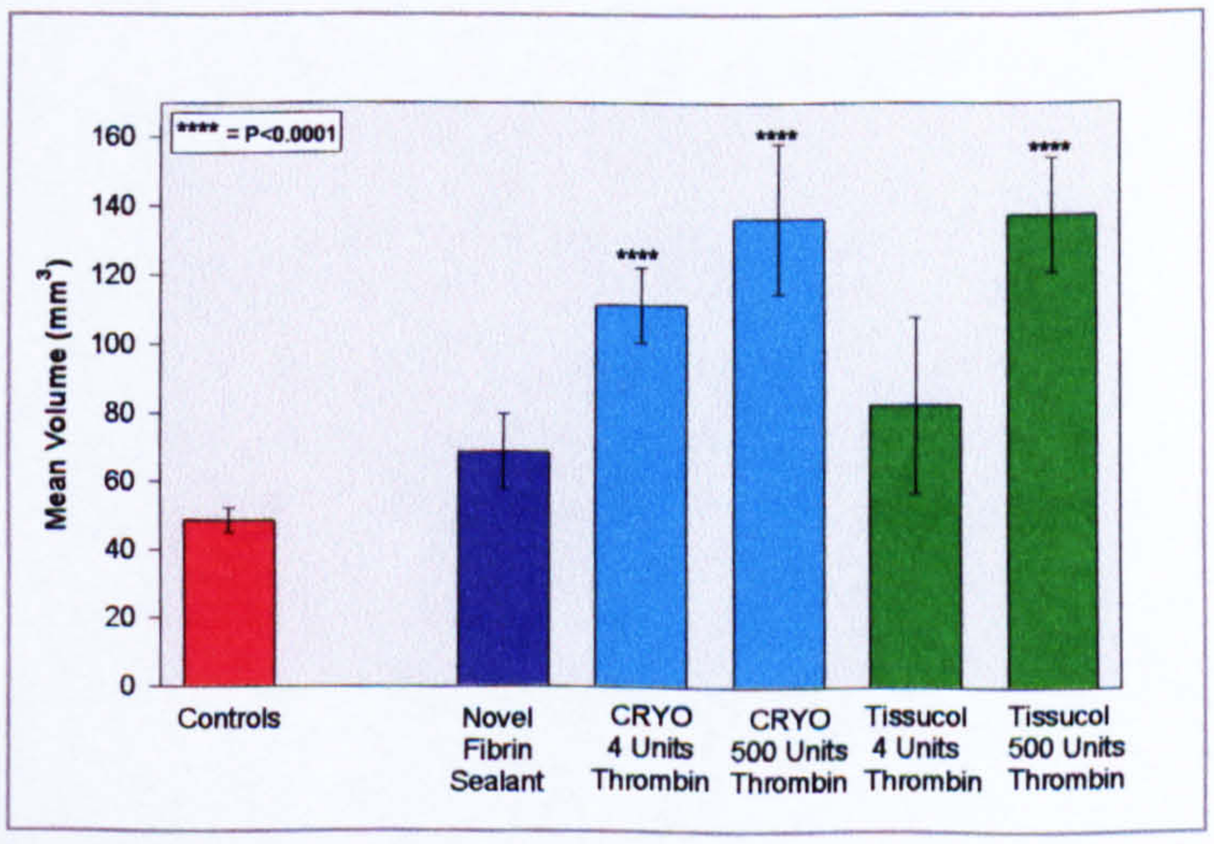
Treatment groups all demonstrated greater mean volumes of peritoneal tissue generated (PTG) compared to controls. Mean PTG for the control group was 48.71mm^3 compared to 68.75 mm^3 for novel fibrin sealant treated, 111.20 and 136.24mm^3 for CRYO treated (4 and 500 units thrombin respectively) and 82.34 and 137.09mm^3 for Tissucol® with 4 and 500 units thrombin respectively (Graph 7.4; Appendix VII Table 7.2). Both CRYO groups and Tissucol® 500 units thrombin demonstrated volumes that were significantly greater than controls ($P<0.001$) (Appendix VII Table 7.3).

Mean volumes of uterine horn tissue generation (UTG) were 12.42mm^3 for the control group, 6.69mm^3 for novel fibrin sealant, 13.95 and 33.95mm^3 for 4 and 500 unit thrombin CRYO and 14.45 and 28.26mm^3 for Tissucol® 4 and 500 units thrombin groups respectively (Graph 7.5; Appendix VII Table 7.2). Mean volumes were significantly

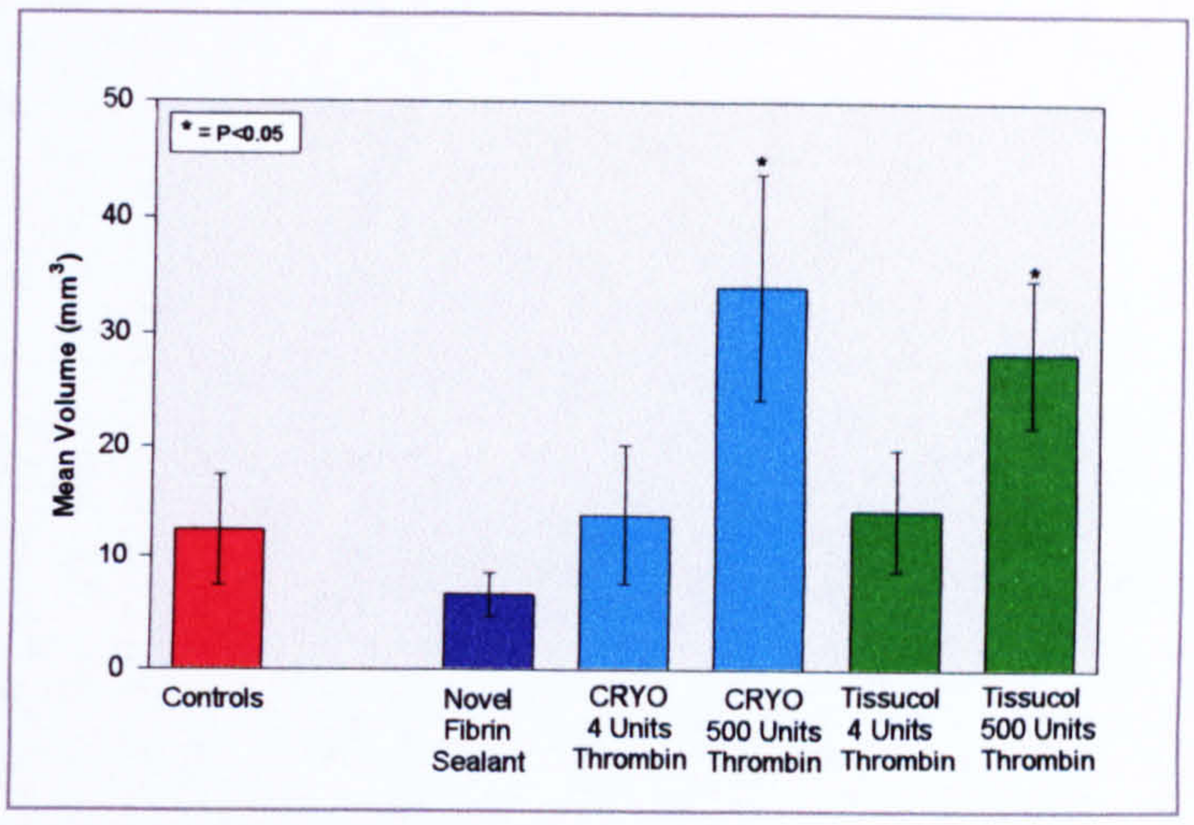
greater ($P<0.05$) than controls in the 500 unit thrombin groups (CRYO and Tissucol®) (Appendix VII Table 7.3).

All treatment groups, except novel fibrin sealant, demonstrated greater mean volumes of total tissue generated (TTG) compared to controls in response to the abrasion injury (Graph 7.6; Appendix VII Table 7.2). The control group showed a mean volume of $136.07mm^3$ compared to $89.11mm^3$ for novel fibrin sealant, 147.70 and $350.94mm^3$ for CRYO (4 and 500 units thrombin respectively) and 204.23 and $238.04mm^3$ for Tissucol® (4 and 500 units thrombin respectively). Both 500 unit thrombin groups (CRYO and Tissucol®) possessed significantly greater ($P<0.05$) volumes of TTG compared to controls, whereas significantly lower ($P<0.05$) volumes were seen with novel fibrin sealant (Appendix VII Table 7.3).

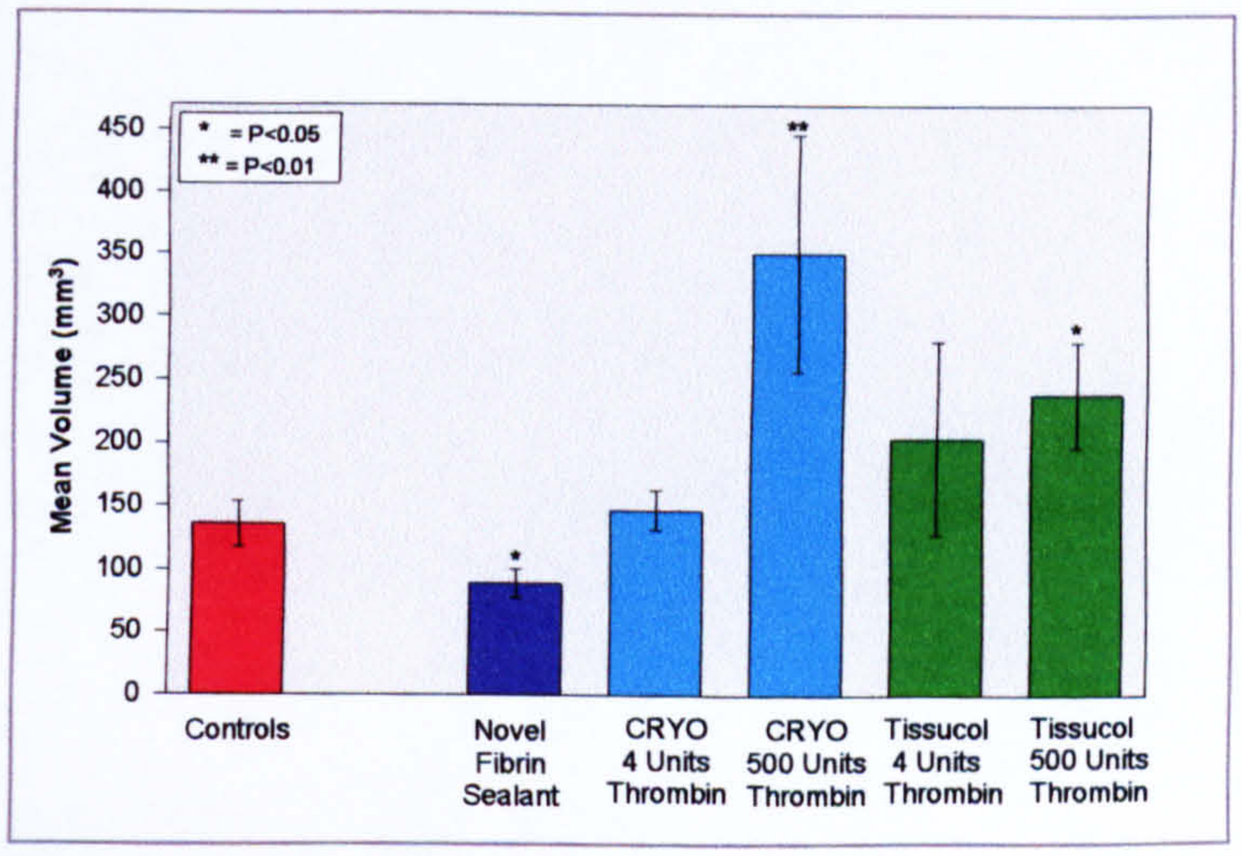
Mean volumes of peritoneal damage were $44.29mm^3$ for controls, $18.02mm^3$ for novel fibrin sealant, 48.78 and $46.13mm^3$ for 4 and 500 units thrombin CRYO and 50.62 and $35.20mm^3$ for Tissucol® treated groups (4 and 500 units thrombin respectively) (Graph 7.7; Appendix VII Table 7.2). Only novel fibrin sealant showed any significant difference from the control group with volumes being significantly lower in the treated group ($P<0.01$) (Appendix VII Table 7.3).



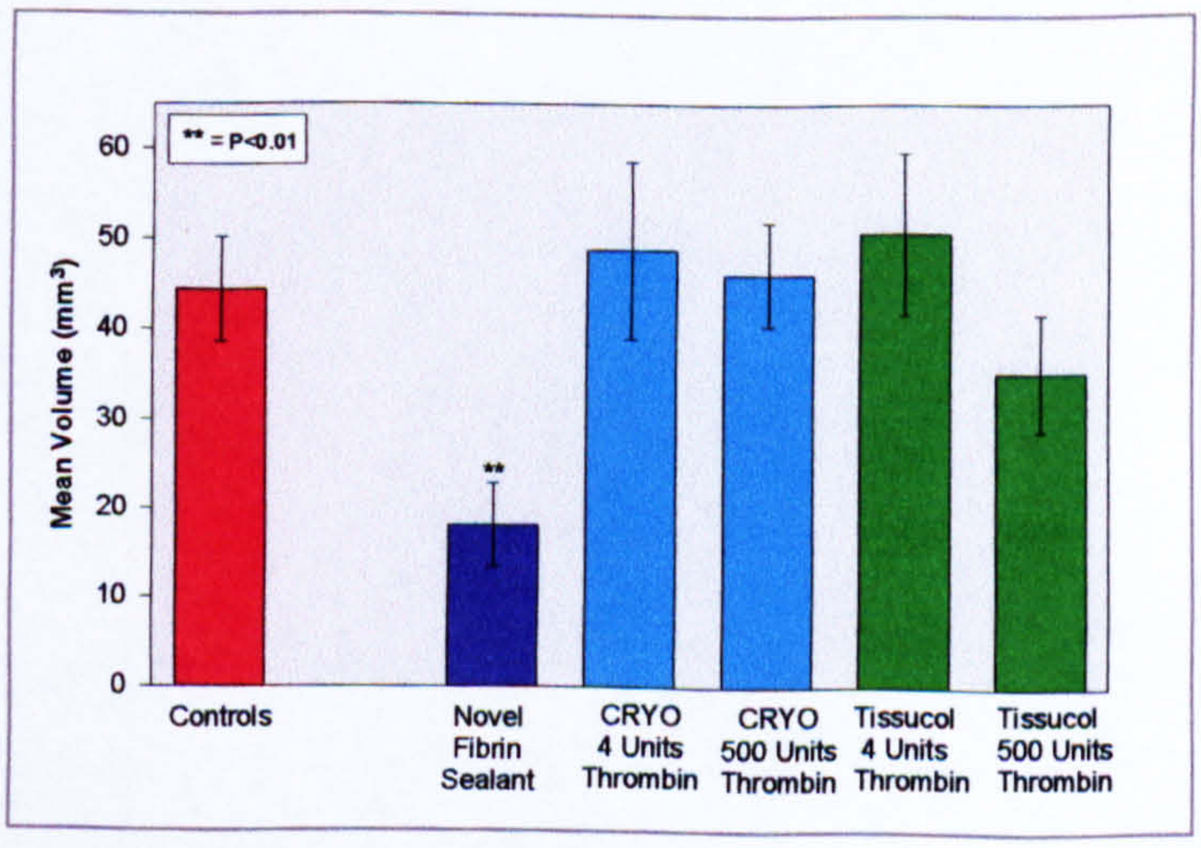
Graph 7.4: Mean PTG volumes
together with SEMs and statistical differences.



Graph 7.5: Mean UTG volumes
together with SEMs and statistical differences.



Graph 7.6: Mean TTG volumes
together with SEMs and statistical differences.



Graph 7.7: Mean peritoneal damage volumes
together with SEMs and statistical differences.

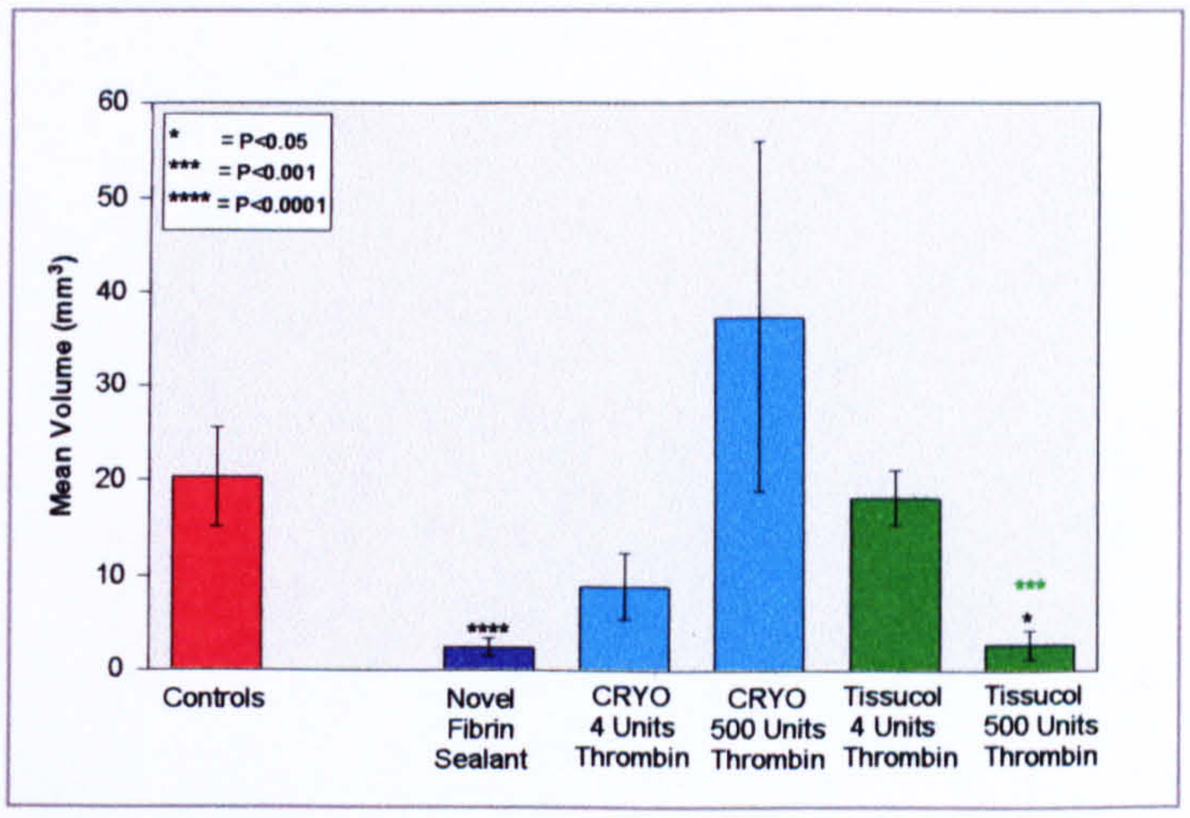
Uterine horn damage mean volumes were 20.26mm^3 for controls, 2.50mm^3 for novel fibrin sealant, 8.89 and 37.38mm^3 for CRYO (4 and 500 units thrombin respectively) and 18.08 and 2.71mm^3 for 4 and 500 units thrombin with Tissucol® (Graph 7.8). Both novel fibrin sealant and Tissucol 500 units thrombin groups had significantly lower mean volumes compared to the control group ($P<0.0001$ and $P<0.05$ respectively). There was also a significant difference ($P<0.01$) between Tissucol® treatments with 4 and 500 units thrombin (Appendix VII Table 7.3).

Mean volume of peritoneal reaction of the control group was 108.62mm^3, compared to 79.58mm^3 for novel fibrin sealant, 88.18 and 174.90mm^3 for CRYO (4 and 500 units thrombin respectively) and 122.09 and 137.72mm^3 for Tissucol® (4 and 500 units thrombin) (Graph 7.9; Appendix VII Table 7.2). The novel fibrin sealant group demonstrated significantly lower ($P<0.01$) volumes of peritoneal reaction compared to controls whereas the CRYO 500 units thrombin group showed significantly greater volumes ($P<0.01$) compared to controls. A significant difference was also observed between CRYO groups ($P<0.0001$), with 500 units demonstrating greater volumes (Appendix VII Table 7.3).

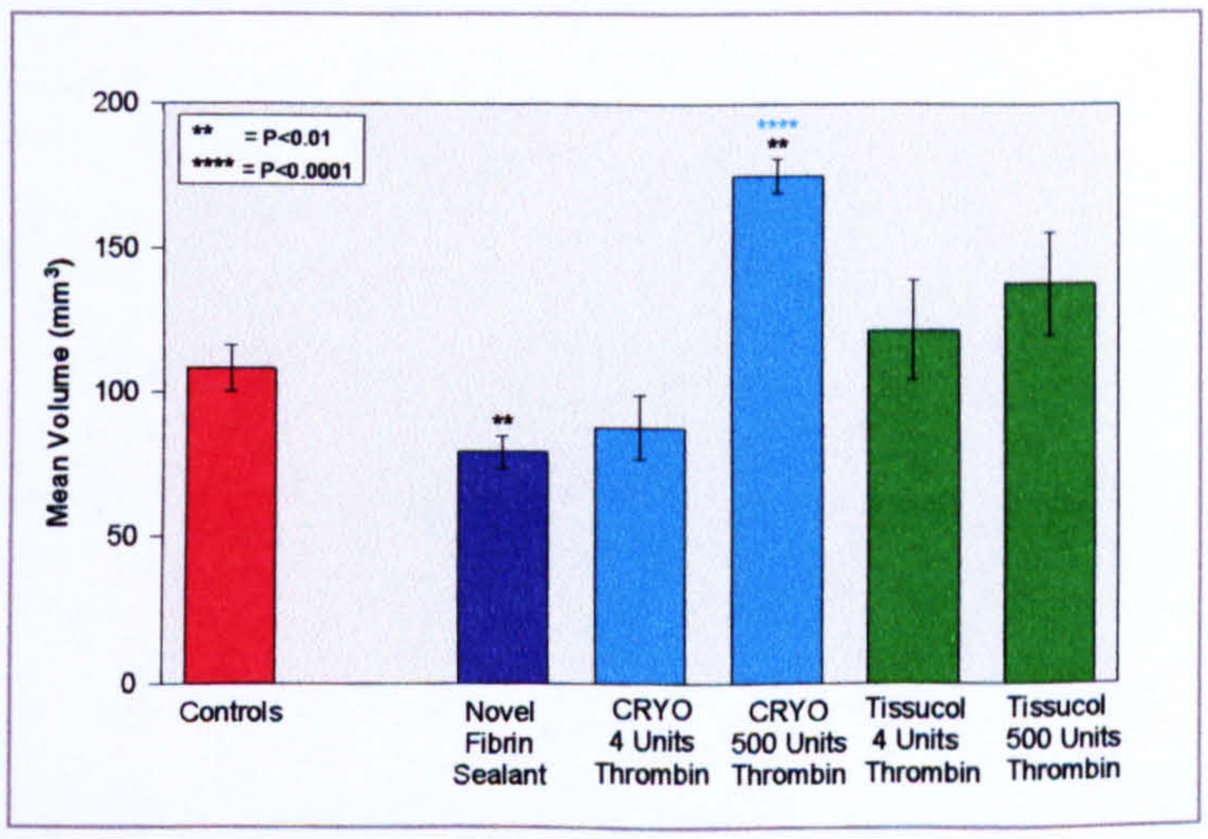
All treatment groups had fibrin sealant remaining at the completion of this 14 day study. Mean volume of novel fibrin sealant was 21.20mm^3, compared to 13.64 and 97.07mm^3 for 4 and 500 units thrombin with CRYO and 42.47 and 110.70mm^3 for 4 and 500 units thrombin with Tissucol® (Graph 7.10; Appendix VII Table 7.2). Significant differences ($P<0.05$) were seen between the novel fibrin sealant groups and the 500 units thrombin groups (CRYO and Tissucol®) and between 4 and 500 units CRYO groups ($P<0.01$) (Appendix VII Table 7.3).

Cell concentration volumes were all significantly greater ($P<0.01$) for all treated groups compared to the control group. Controls demonstrated 1.61mm^3, compared to novel fibrin sealant with 29.62mm^3, CRYO 22.87 and 79.16mm^3 (4 and 500 units thrombin respectively) and Tissucol® 35.33 and 72.48mm^3 (4 and 500 units thrombin respectively) (Graph 7.11; Appendix VII Table 7.2). A significant difference ($P<0.01$) was seen

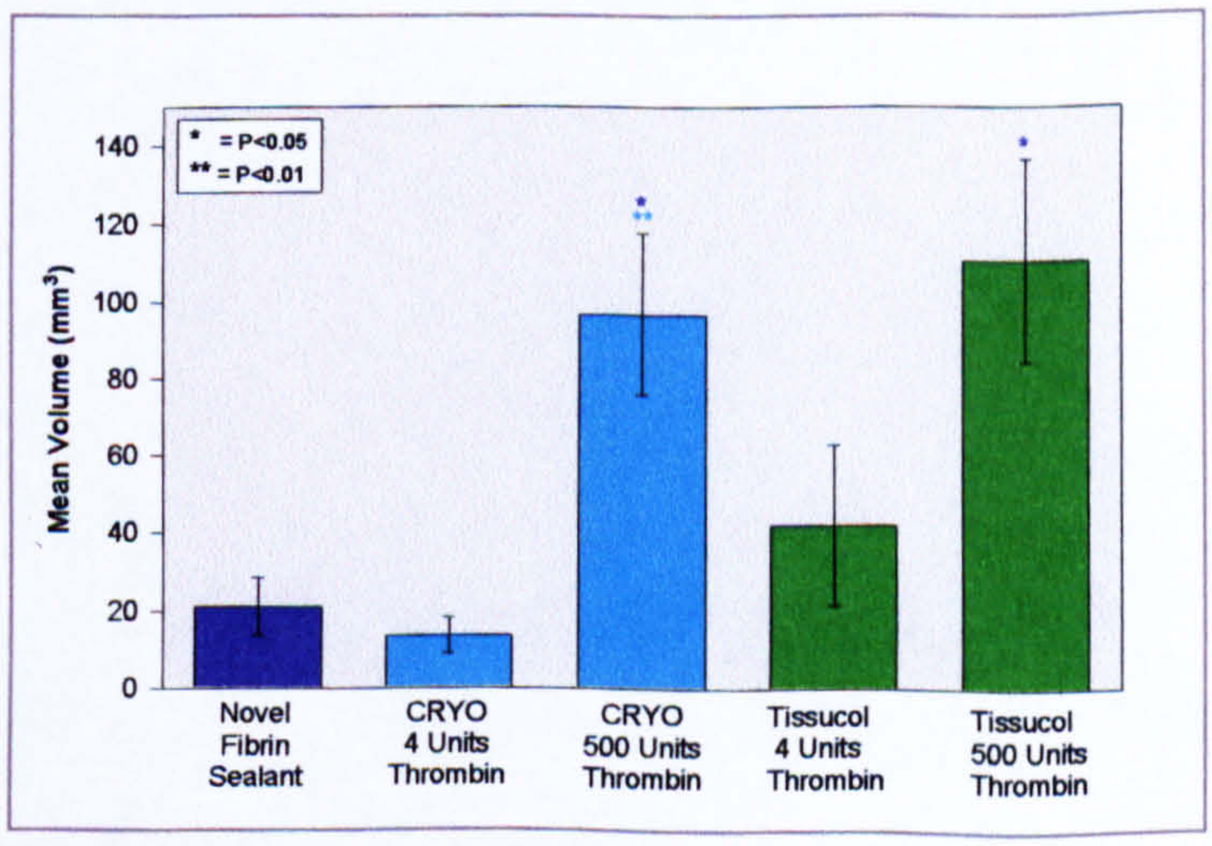
between 4 and 500 units thrombin with CRYO (Appendix VII Table 7.3).



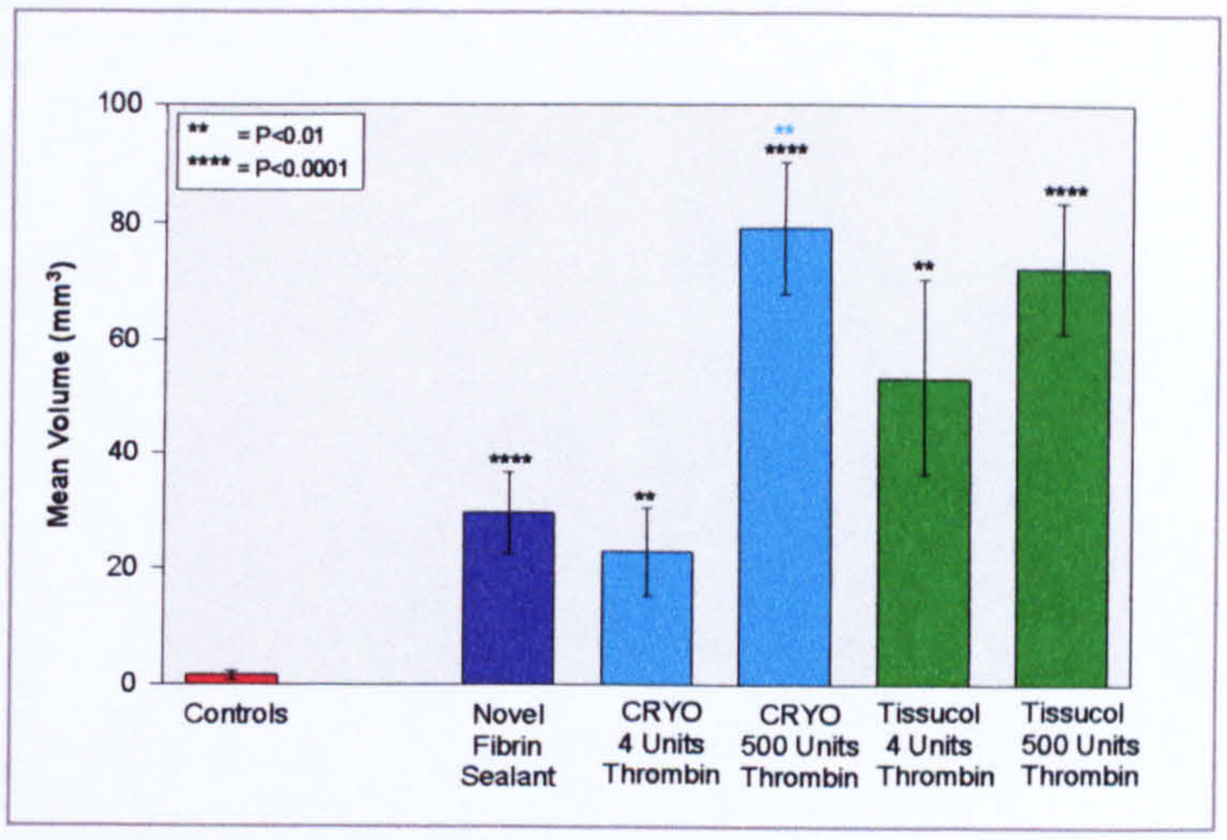
Graph 7.8: Mean uterine horn damage volumes
together with SEMs and statistical differences.



Graph 7.9: Mean peritoneal reaction volumes
together with SEMs and statistical differences.



Graph 7.10: Mean fibrin sealant remaining volumes
together with SEMs and statistical differences.



Graph 7.11: Mean cell concentration volumes together with SEMs and statistical differences.

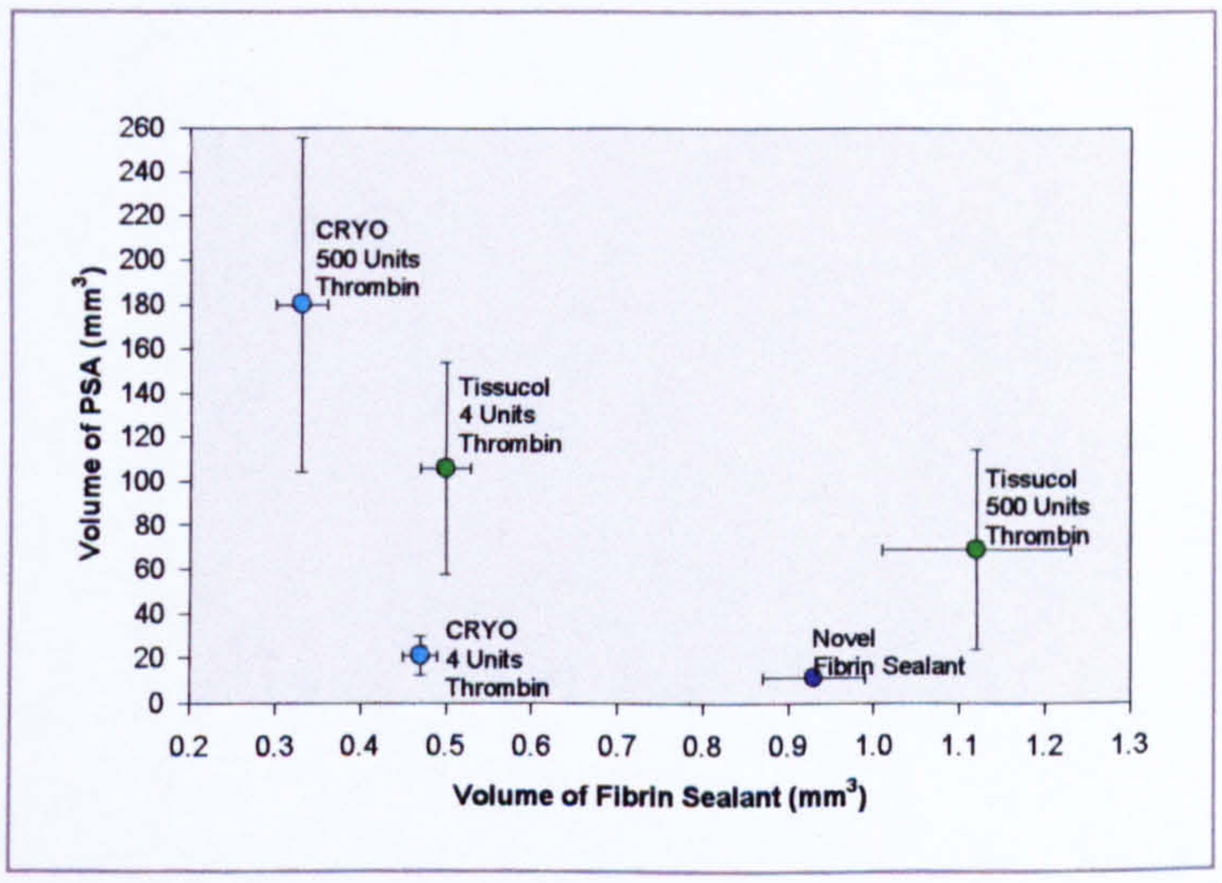
7.4 Discussion

This study utilised a definitive rabbit uterine horn abrasion model together with quantitative analysis to assess PSA reduction and/or prevention using novel fibrin sealant, Tissucol® and CRYO. Since the same PSA induction procedure and unbiased and precise quantitative analytical procedures were used for all groups, fair and valid comparisons can be made.

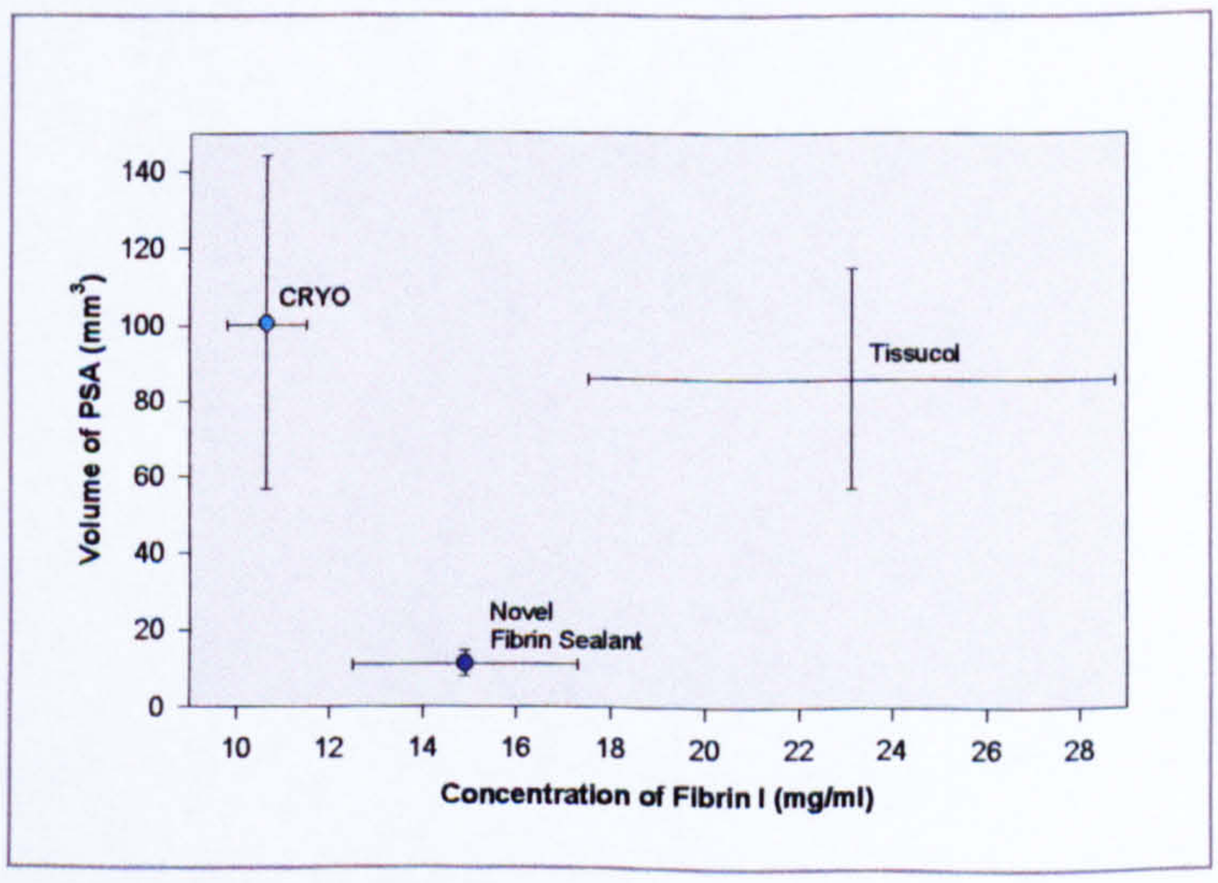
Only two of the treatments investigated, novel fibrin sealant ($P<0.001$) and CRYO with 4 units thrombin ($P<0.05$), significantly reduced the formation of PSAs. Tissucol® with 500 units thrombin showed only a 6% reduction in PSAs however Tissucol® 4 units thrombin and CRYO 500 units thrombin groups demonstrated an increase in the volume of PSAs present, with an additional 44 and 144% respectively. Hence Tissucol® 4 and 500 units thrombin and CRYO 500 units thrombin did not significantly reduce either the incidence or volume of PSAs compared to controls. These results are in contradiction to previously published papers supporting the use of Tissucol® (Lindenberg & Lauritsen, 1984; Lindenburg *et al.*, 1985; Larsson *et al.*, 1986; De Laco *et al.*, 1994) and CRYO (CRYO: Sheppard *et al.*, 1993; Ethanol precipitate: Weiss-Fogh *et al.*, 1990) as PSA preventative agents. As with all potential PSA preventative agents, previous studies investigating Tissucol® have used a wide variety of experimental models coupled with

subjective qualitative analytical techniques. Since this study eliminated the problems associated with these investigation methods, it is conclusive that Tissucol® (4 and 500 units thrombin) and CRYO (500 units thrombin) do not significantly reduce/prevent PSAs in this rabbit uterine horn abrasion model. This gives support to studies which also do not support the use of Tissucol® as an anti-PSA agent (Bilgin *et al.*, 1995).

Effectiveness of PSA prevention by fibrin sealants appears to be independent of the volume applied (Graph 7.12) and fibrin I concentration (Graph 7.13) within the limits used in this study. Of the fibrin sealants investigated, novel fibrin sealant demonstrated little variation in PSA volume even with larger variations in volume applied and fibrin I concentration.



Graph 7.12: Mean PSA volume against mean volume of fibrin sealant applied, together with SEMs.



Graph 7.13: Mean PSA volume against mean concentration of fibrin sealant applied, together with SEMs.

With the exception of UTG present in the novel fibrin sealant group, all treatment groups demonstrated greater volumes of PTG and UTG compared to controls. In both 500 units thrombin groups (CRYO and Tissucol®) this increase in mean volumes of PTG and UTG was significant ($P<0.05$) compared to controls. It is generally assumed that one of the mechanisms in which fibrin sealants prevent PSAs is by acting as a physical barrier, preventing fibrin exuded from damaged tissues from adhering to other surfaces, thus preventing the formation of fibrinous and subsequently fibrous adhesions. If fibrin sealants are acting solely as a physical barrier, with no biochemical actions, then injured surfaces should produce the same volume of fibrin in response to injury but this will be discretely located on injured surfaces rather than joining them together. The increase in PTG and UTG in treatment groups compared to controls supports this hypothesis.

To substantiate this theory the mean total tissue generated from treated and control groups should be similar. However this is only true for 4 units thrombin groups (CRYO and Tissucol®). The novel fibrin sealant group demonstrated a significantly lower ($P<0.05$) mean volume of TTG compared to controls, indicating physiological modulation occurring to reduce the amount of tissue generated in response to injury. Results from 500 unit thrombin groups (CRYO and Tissucol®) also indicate some type of modulation, however in these groups there is significant ($P<0.05$) stimulation of tissue generated. This phenomena of tissue fibrosis by thrombin dependent fibrin sealants has been highlighted by other authors, especially with nerve fibrosis (Fairbrother, 1997). Increased stimulation of fibrotic tissue in response to damage is not a desirable effect of a fibrin sealant, since the greater volume of tissue generated the greater risk of problems associated with that tissue.

None of the treatments except novel fibrin sealant had any effect on the mean volume of both peritoneal and uterine horn damage, which demonstrated a significant decrease compared to controls ($P<0.01$ and $P<0.0001$ respectively). Since all experimental animals underwent the same injury process, that is, a similar volume of initial injury within biological limits, then this significant decrease seen in mean damage volumes with

novel fibrin sealant indicates a limitation in subsequent tissue damage, that is, necrosis of underlying and adjacent tissues. This is further evidence that novel fibrin sealant exerts physiological regulation of tissue repair with further support from the significant decrease ($P<0.01$) in mean volume of peritoneal reaction compared to controls. It is hypothesised that novel fibrin sealant limits the inflammatory response mounted in response to tissue damage, either due to tissue isolation from external factors or by biochemical modulation, which in turn reduces the amount of damage induced by the inflammatory response, and therefore the amount of tissue generated in response to damage. Many studies have demonstrated that there is a direct relationship between PSA formation and the extent of tissue damage (Ellis, 1962; Belzer, 1967) and hence the reduction in damage volume with novel fibrin sealant is most probably associated with the reduction seen in PSAs and TG.

Mean peritoneal reaction volumes of all of the other treatments (except CRYO 500 units thrombin) showed no change from controls, indicating that they do not influence the tissue reaction mounted in response to injury. The significant increase ($P<0.01$) in peritoneal reaction seen with CRYO 500 units thrombin is due to the increased thrombin concentration used, since 4 units thrombin showed no such difference and there was a significant increase ($P<0.0001$) between 4 and 500 units thrombin.

High concentrations of thrombin *per se* have several effects including macrophage migration into the fibrin clot, reduction or inhibition of fibroblast migration into the clot, reduction or inhibition of fibroblast attachment and mitogenesis, induction of lung injury, brain oedema and cerebral vasospasm, vascular thrombogencity and fibrosis in nerve repair (Fairbrother, 1997). Although thrombin is not added directly into areas treated with thrombin dependent fibrin sealants, it can enter the body via inadequate mixing of the sealant and release from the sealant matrix. The above adverse effects are potentially possible with the use of these fibrin sealants. From this study it is clear that some of these effects can be seen, specifically increased cellular migration into injured tissue and excessive tissue fibrosis.

Tissucol® has been designed to use one of two thrombin concentrations, 4 and 500 units. The use of 4 units thrombin has been recommended for all tissue adaptation applications, for example, skin grafting and microsurgical operations, with the high thrombin concentration suggested for haemostasis to ensure instantaneous clotting (Fairbrother, 1997). However due to the slower clotting time of the lower thrombin concentration many surgeons have tended to use the high concentration thrombin and ignore potential problems associated with the higher concentration.

The concentration of thrombin utilised in fibrin sealants has generally focused on the optimal concentration required for rapid cleavage of fibrinogen to fibrin and subsequent cross-linking of the fibrin II rather than on safety. However the disadvantages of using high thrombin concentration are beginning to be noticed and the trend for the use of lower thrombin concentrations in fibrin sealants has begun to emerge. Unlike other fibrin sealants, no exogenous thrombin is required for novel fibrin sealant polymerisation, with the sealant containing less than 1 unit of endogenous thrombin. The results from this study highlight the advantages of such a system over traditional fibrin sealants.

All treatment groups demonstrated fibrin sealant still remaining at the completion of this 14 day study. There were significant differences ($P<0.05$) seen between 500 units thrombin for both CRYO and Tissucol® compared to novel fibrin sealant, and between 4 and 500 units thrombin with CRYO. It appears that the addition of 500 units thrombin for fibrinogen cleavage significantly increases the residency time of the resulting fibrin sealant *in vivo*. This does not appear to be directly related to the volume of fibrin sealant added, but could be due to increased fibrinolytic resistance of the sealant or increased amount of fibrinogen polymerised by the faster acting 500 units of thrombin.

The addition of exogenous proteins to the peritoneal cavity is highly probable to induce a foreign body reaction. This was indeed the case with all fibrin sealant treatment groups demonstrating significantly greater ($P<0.001$) volumes of cell concentration compared to controls. Although novel fibrin sealant is intended as an autologous fibrin sealant, this is naturally not the case in this study since human derived novel fibrin sealant was used to

treat injuries in the rabbit peritoneum.

7.5 Conclusions

i. PSAs were significantly reduced by:

   a. Novel fibrin sealant - 85% reduction

      It is hypothesised that this is due to:

      - it acting as a physical barrier, separating and protecting injured surfaces from external influences, and/or
      - biochemical modulation (limiting the inflammatory response to injury, decreasing damage volume and volume of tissue generated).

   b. CRYO 4 units thrombin - 72% reduction

      It is hypothesised that this due to:

      - it acting as a physical barrier, separating and protecting injured surfaces from external influences

ii. No significant effect on PSAs was seen with:

   a. CRYO 500 units thrombin - 144% increase
   b. Tissucol® 4 units thrombin - 44 % increase
   c. Tissucol® 500 units thrombin - 6% reduction

iii. High exogenous thrombin concentrations (500 units) exerted several physiological effects:

   - significantly increased volumes of total tissue generated
   - increased volumes of peritoneal reaction
   - significantly increased cell concentration volumes
   - increased the residence time of fibrin sealants *in vivo*

iv. All non-autologous fibrin sealants induce a cell mediated foreign body reaction *in vivo*. The magnitude of this reaction was increased with exogenous thrombin concentration.

v. Novel fibrin sealant demonstrated unique actions compared to the other fibrin sealants investigated - significant reduction ($P<0.05$) in volumes of PSAs, TTG, damage and peritoneal reaction. Hence novel fibrin sealant reduces and prevents PSA formation and modulates tissue repair.

vi. In this direct comparison study, novel fibrin sealant was more effective and safe than Tissucol® or CRYO (4 and 500 units thrombin) for the prevention of PSAs in this rabbit uterine abrasion model.

8.0 Pathogenesis of PSAs and Prevention with Vivostat™ System Human and Porcine Derived Fibrin Sealant in the Pig Abrasion Model - Pilot Study

8.1 Introduction

All studies investigating the effect of Vivostat™ system derived human fibrin sealant (novel fibrin sealant) on PSA development using the rabbit uterine horn model have demonstrated significant reduction to near complete prevention of PSAs (refer to Chapters 4, 6 and 7). To date the rabbit is the only species utilised for prevention of PSAs with novel fibrin sealant. For regulatory approval of a product for clinical trials effectiveness and safety needs to be demonstrated in two distinct animal species. Thus a second species, pig, was selected for investigation due to its physiological similarities to man and also its large size. Although previous rabbit experimental studies have good clinical representation they do not utilise novel fibrin sealant as intended, that is, as a truly autologous fibrin sealant. In order for this to occur blood needs to be taken from a subject (animal or human), novel fibrin sealant prepared and then used to treat that subject. This is unattainable using small experimental models, such as the rabbit, but attainable with a larger porcine experimental model.

The aims of this study were to:

i. assess the ability of human and porcine derived novel fibrin sealant to reduce PSAs in a porcine abrasion model using quantitative analysis.
ii. identify possible mechanisms of PSA prevention.
iii. compare human and porcine derived novel fibrin sealant for PSA prevention in a porcine model.

8.2 Materials and Methods

This pilot study comprised of 3 experimental groups (Table 8.1) all recovered 7 days post operative. Surgical and termination procedures were as described in Chapter 2. Volume of novel fibrin sealant applied to each experimental site was left to the discretion of the surgeon and ranged between 1.2 and 2.0ml per site (Table 8.1).

Treatment Group	Number of Animals	Injury Site	Number of Experimental Sites	Volume of fibrin sealant Applied (ml)	Fibrin I Conc^{n} (mg/ml)
Control	3	Stomach Colon Caecum	3 4 2 Total : 9	N/A	N/A
Human novel fibrin sealant	3	Stomach Colon Caecum	2 4 2 Total: 8	1.56 ± 0.06	18.01 ± 2.29
Porcine novel fibrin sealant	2	Stomach Colon Caecum	1 2 2 Total: 5	1.25 ± 0.05	19.45 ± 2.93

Table 8.1: Study summary.

Formalin fixed resected tissues were uniform randomly sliced transversely, with all slices processed to paraffin wax. 5µm sections were cut from each block, stained with haematoxylin and eosin and analysed using Cavalieri's principal for determining absolute volumes.

Due to small sample size for each injury site statistical analysis was not performed.

8.3 Results

There was one mortality during this study, a porcine novel fibrin sealant treated animal. Post mortem revealed the cause of death to be strangulated hernia due to suture breakdown. There was no other morbidity or mortality during the course of this study.

8.3.1 Macroscopic Results

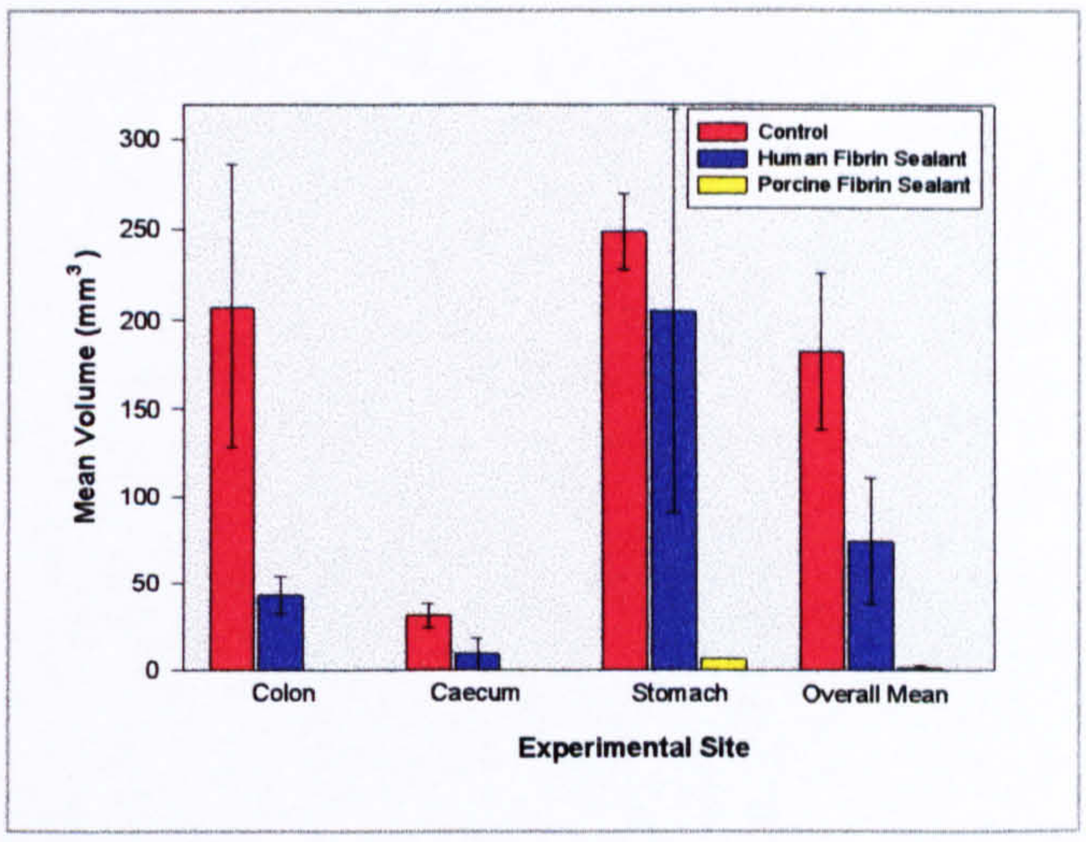
All animals demonstrated general PSAs between the peritoneal suture line and underlying tissues: most frequently the omentum, ileum, caecum, spleen and liver respectively. All these PSAs were separated by blunt dissection and did not interfere with experimental sites.

All control experimental sites were adhered, many being quite severe. 5 out of the 8 sites treated with human novel fibrin sealant were clear of adhesions, with the remaining sites joined by either PSAs or fibrin sealant. 2 out of the 4 porcine novel fibrin sealant treated sites were identified as non-adhered with the other sites joined by either PSAs or fibrin sealant.

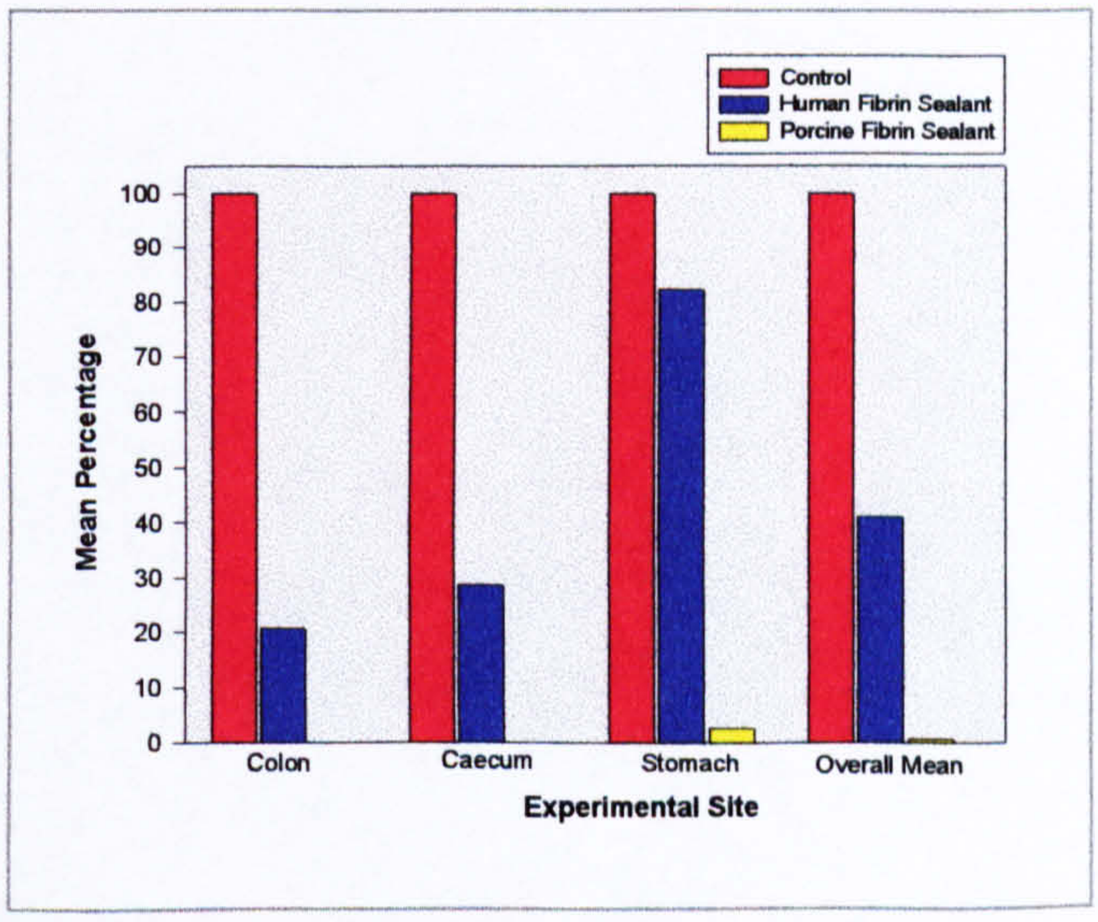
8.3.2 Microscopic Quantitative Analysis

Mean volume of PSAs for the control group was 207.25mm^3 for the colon, 31.33mm^3 for the caecum (Figure 8.1) and 248.11mm^3 for the stomach (Figure 8.2), with an overall mean of 181.78mm^3. Human novel fibrin sealant treated group demonstrated mean volumes of 42.83mm^3, 8.96mm^3 and 204.47mm^3 for the colon, caecum (Figure 8.3) and stomach (Figure 8.4) respectively, with an overall volume of 74.77mm^3. Whereas no PSAs were present in colon and caecum (Figure 8.5) experimental sites treated with porcine novel fibrin sealant, with 6.25mm^3 for the stomach (Figure 8.6) and a group mean of 1.27mm^3 (Graph 8.1; Appendix VIII Table 8.1).

Assuming that the control group demonstrated 100% volume of PSAs, reduction with human novel fibrin sealant was 79, 71 and 18% for the colon, caecum and stomach respectively, with an overall reduction of 59%. Porcine novel fibrin sealant demonstrated 100% reduction for both colon and caecum, 97% for the stomach and overall 99% reduction of PSAs compared to controls (Graph 8.2; Appendix VIII Table 8.1).



Graph 8.1: Mean PSA volumes together with SEMs.



Graph 8.2: Mean PSA percentages.

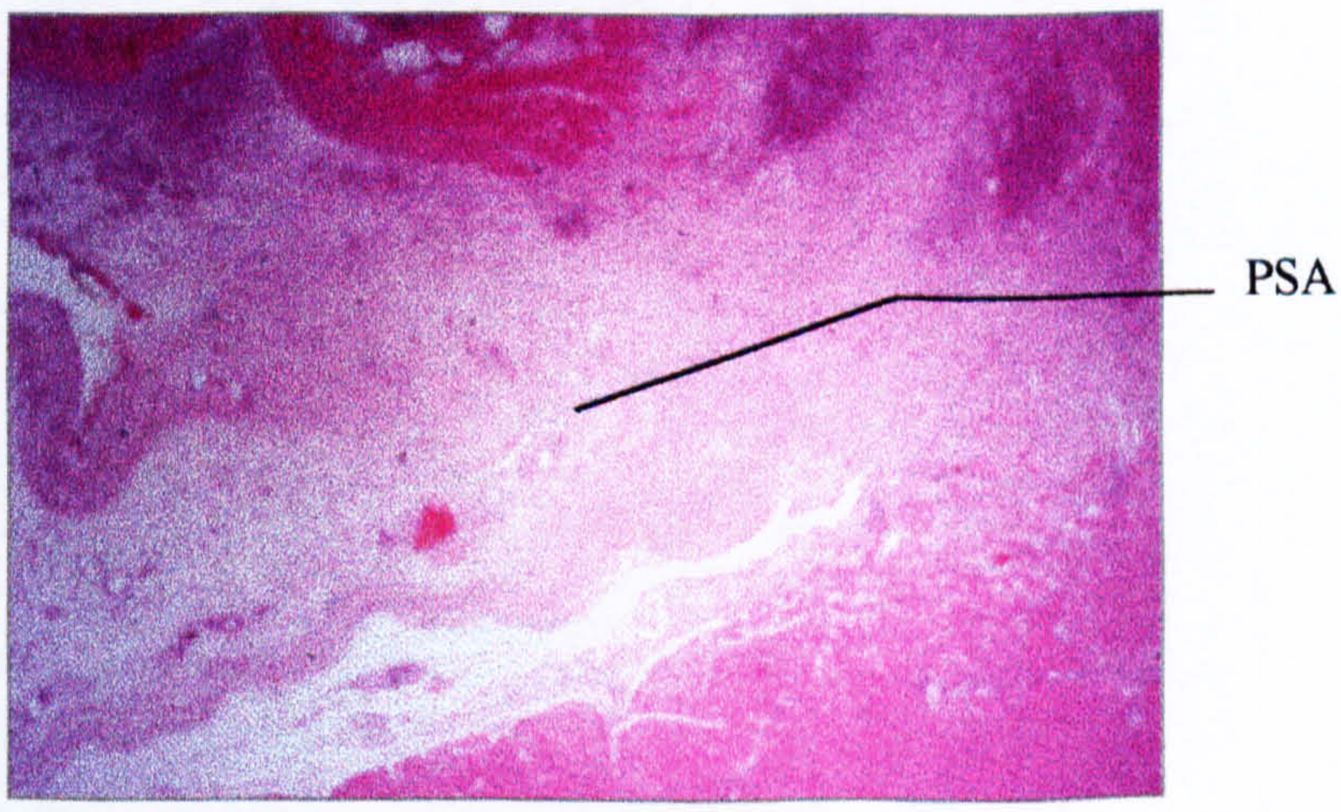


Figure 8.1: Control case caecum. (M_o x2)

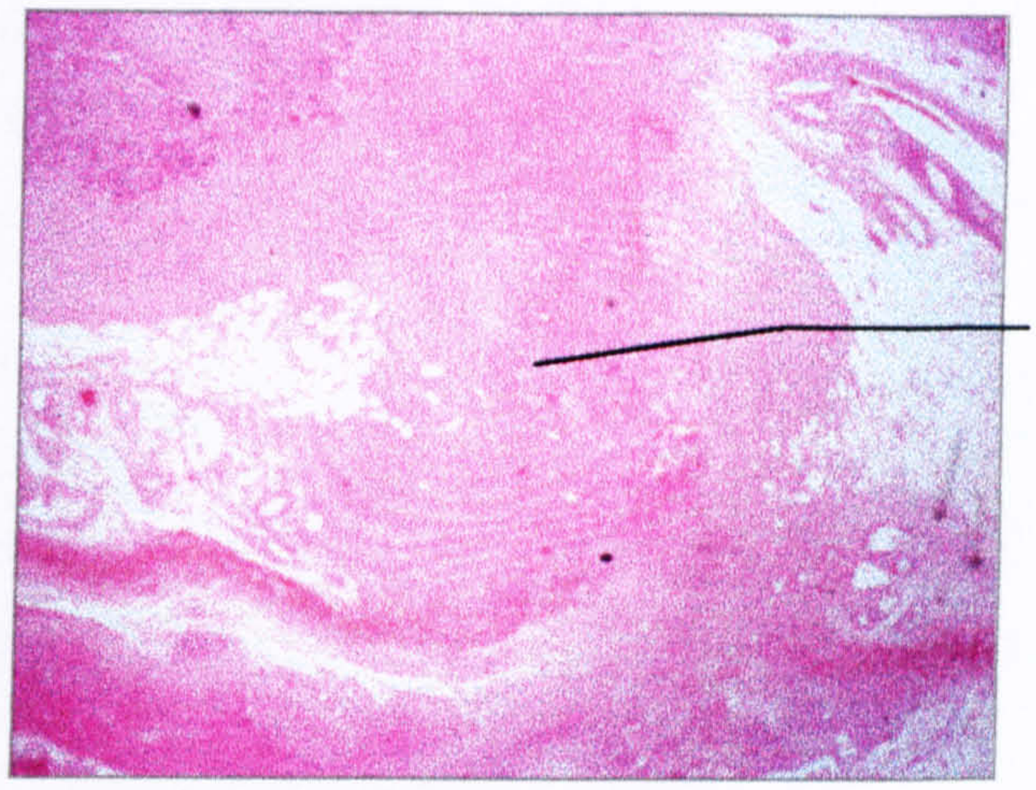


Figure 8.2: Control case stomach.
(M_o x2)

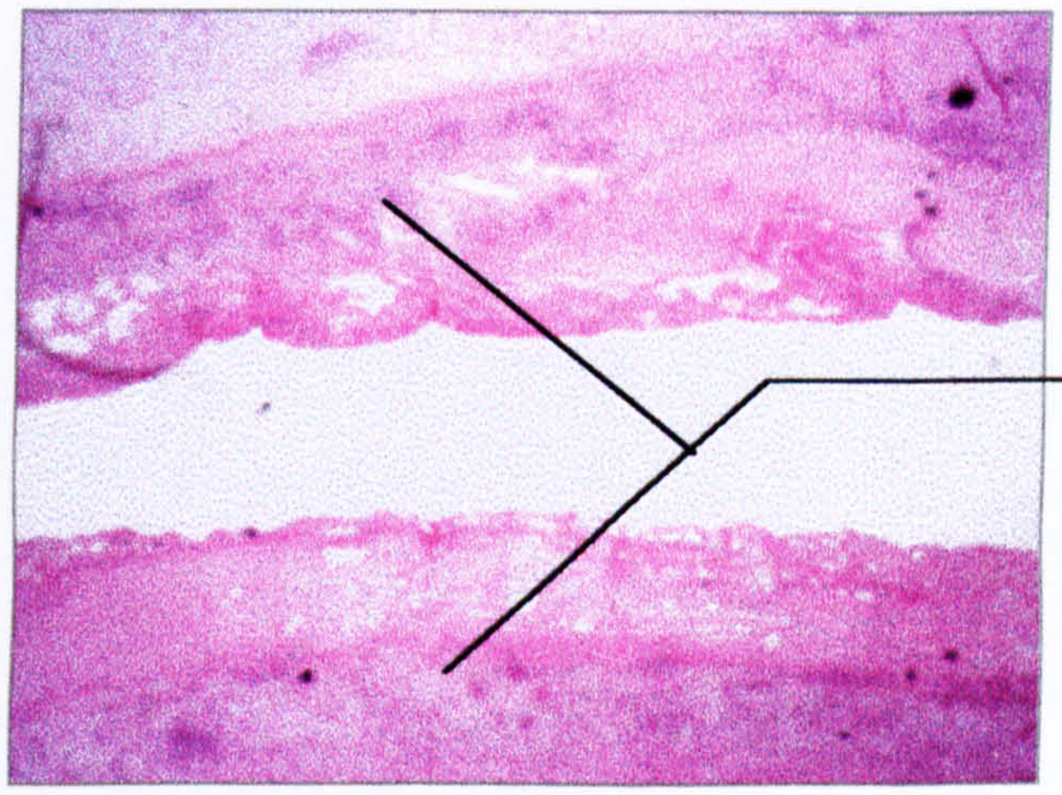


Figure 8.3: Human fibrin sealant treated caecum.
(M_o x2)

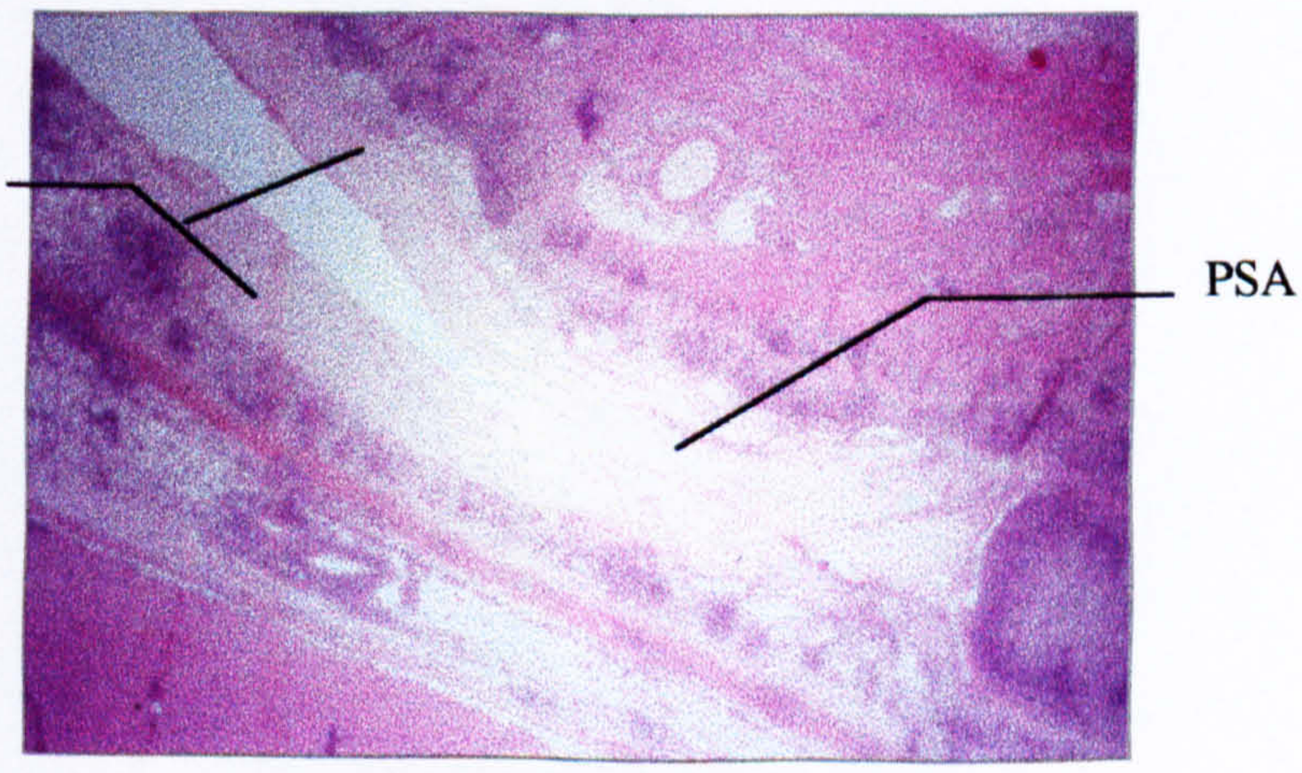


Figure 8.4: Human fibrin sealant treated stomach.
(M_o x2)

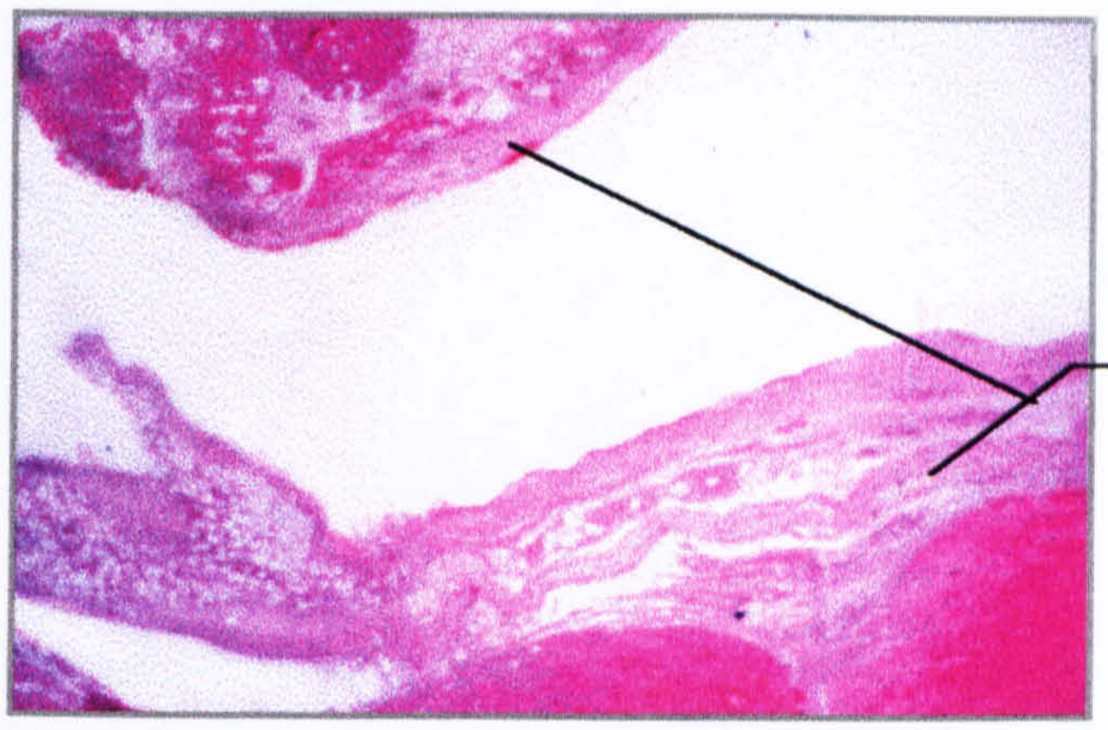


Figure 8.5: Porcine fibrin sealant treated caecum.
(M_o x2)

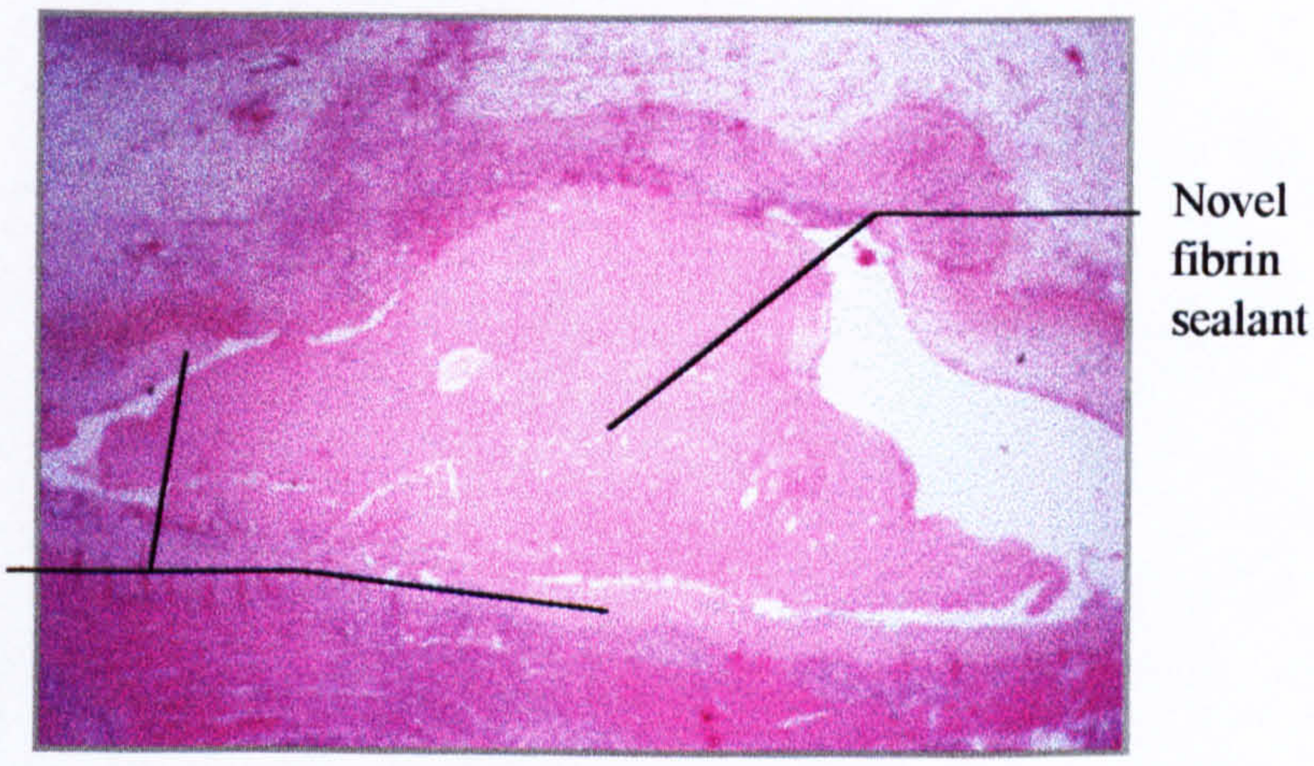


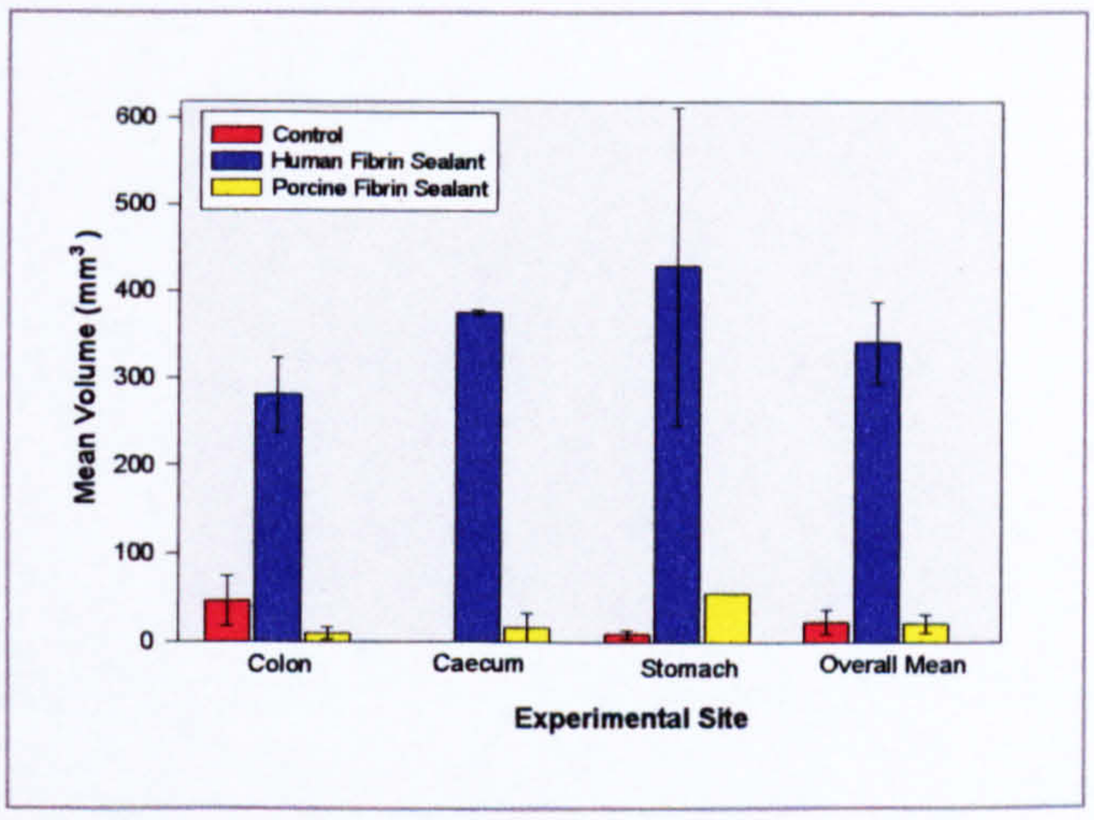
Figure 8.6: Porcine fibrin sealant treated stomach.
(M_o x2)

All experimental sites treated with human novel fibrin sealant demonstrated much greater mean volumes of inflammation compared to control or porcine novel fibrin sealant groups (Graph 8.3; Appendix VIII Table 8.1). Mean volumes of inflammation for control sites were 45.54, 0.00, 9.01 and 23.24mm^3 for the colon, caecum, stomach and overall mean and for porcine derived novel fibrin sealant they were 9.17, 16.28, 55.24 and 21.23mm^3 for colon, caecum, stomach and overall mean. However, for human derived novel fibrin sealant mean inflammation volumes were 280.20, 375.02, 429.27 and 341.17mm^3 for colon, caecum, stomach and overall mean.

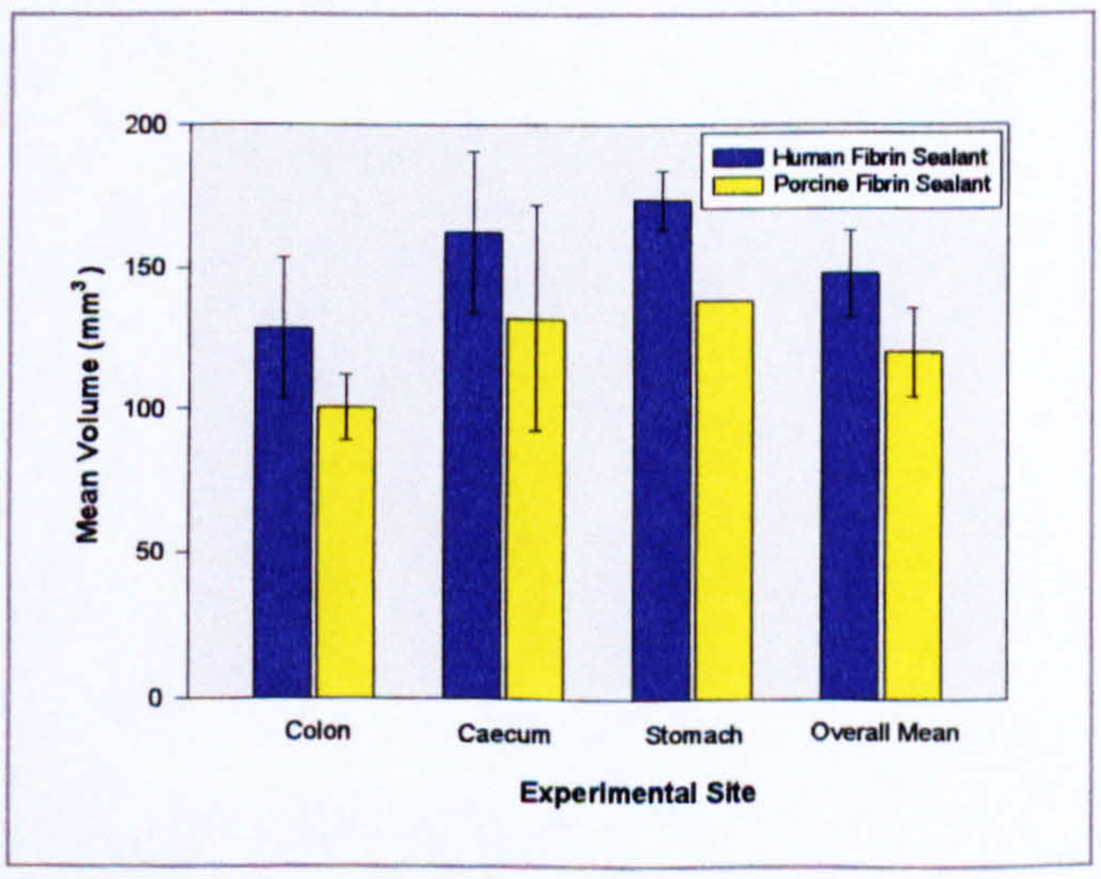
All experimental sites treated with either human or porcine fibrin sealant demonstrated fibrin sealant remaining at the end of this 7 day study (Graph 8.4; Appendix VIII Table 8.1). Human novel fibrin sealant sites had mean volumes of 128.87, 162.82, 174.12 and

148.67mm^3 at the colon, caecum, stomach and overall mean respectively. Sites treated with porcine novel fibrin sealant demonstrated mean volumes of 100.45, 132.38, 139.23mm^3 for colon, caecum and stomach sites respectively with an overall mean for the group of 120.98mm^3.

The coefficient of error for this study was calculated to be 12%.



Graph 8.3: Mean inflammation volumes together with SEMs.



Graph 8.4: Mean novel fibrin sealant remaining volumes together with SEMs.

8.4 Discussion

Treatment of abraded colon, caecum or stomach apposed with abraded peritoneum, with human or porcine novel fibrin sealant reduced both the incidence and severity of PSAs.

Overall reduction of PSAs with human novel fibrin sealant was 59% and with porcine novel fibrin sealant 99%. There are several possible reasons for the differences seen between PSA reduction using human and porcine novel fibrin sealant in this experimental model:

i. It is well recognised that there is a relationship between volume of damage and extent of PSAs formed (Ellis, 1962; Belzer, 1967; Chapter 3). Hence differing PSA reductions could be due to differences between the extent of initial and subsequent damage sustained by treated sites, with greater volumes in those treated with human compared to porcine fibrin sealant. However the probability of such differences is low since all animals underwent the same standardised injury process to induce PSAs and therefore sustained the same extent and volume of original damage within biological variation.

ii. More effective application of porcine novel fibrin sealant compared to human derived fibrin sealant. Again this is unlikely since application of both fibrin sealants was carried out using the Vivostat™ spray applicator and, although variations do occur with regard to application quality, these were not marked during this study.

iii. Differing fibrin I concentrations for porcine and human preparations. The Vivostat™ processor is designed to produce final fibrin I concentrations of approximately 20mg/ml, altering the final volume of fibrin I in order to achieve this objective. Hence any variations in fibrin I concentration are minimal which was the case in this study, with mean concentrations of 18.01±2.29mg/ml for human fibrin I and 19.45±2.93mg/ml for porcine fibrin I.

iv. Truly autologous fibrin sealants possess greater PSA reduction properties compared to non autologous fibrin sealants. This is the most probable explanation for the differences observed and is highlighted by volumes of inflammation present with the human novel sealant group being 15 fold greater than controls whereas pig novel fibrin sealant groups demonstrated no difference. The inflammatory response is hypothesised to be an immune mounted response due to the introduction of human protein into the pig peritoneum. Although both incidence and severity of PSAs were reduced, this increased inflammatory response will exacerbate the inflammatory response which naturally occurs in response to injury, thus delaying and potentially

hampering the progression of wound healing. Hence PSAs are probably not maximally prevented with non-autologous fibrin sealant.

The site of PSA induction demonstrated an effect on the volume of PSA generated, with the stomach having the highest mean volume, followed by the colon and finally the caecum. With such a small number of experimental sites it is not possible to say whether these are significantly different, however the lower mean volumes seen in the colon and caecum could be due to:

i. motility of the bowel leading to some fibrinous PSAs being disrupted, and hence a smaller volume of fibrous adhesions
ii. difference in fibrinolytic capacity between the bowel and stomach (Marshall, 1985).
iii. stomach sustaining a greater volume of underlying and adjacent damage during the injury process, with the well recognised relationship between PSAs and damage (Ellis, 1962; Belzer, 1967; Chapter 3 of this thesis) resulting in a greater volume of PSAs.

With regard to novel fibrin sealant stability *in vivo*, both human and porcine derived sealant was present at the conclusion of this 7 day study. Similar volumes were present in the two groups with a trend for lower volumes with porcine fibrin sealant. Hence the autologous nature of the fibrin sealant appears to play only a minor role in its clearance, at least at 7 days post injury.

Conclusions

1. Treatment with human or porcine fibrin sealant reduced both the incidence and severity of PSAs in this definitive porcine abrasion model.
2. In this porcine model the overall reduction of PSAs with human novel fibrin sealant was 59% and with porcine novel fibrin sealant 99% with the difference in efficiency due to the autologous nature.
3. Experimental sites investigated demonstrated differing PSA induction capacities using the same PSA induction technique, with stomach having greatest PSA volumes, followed by colon and finally caecum.
4. Both human and porcine derived fibrin sealant was present at 7 days post injury with

clearance *in vivo* only mildly affected by the autologous nature of the fibrin sealant at this time.

5. Truly autologous fibrin sealant has greater PSA prevention potential compared to non-autologous fibrin sealant.

9.0 Pathogenesis of PSAs and Prevention with Vivostat™ System Human Derived Fibrin Sealant in the Pig Abrasion Model - Main Study

9.1 Introduction

Previous studies have demonstrated that Vivostat™ system human derived fibrin sealant (novel fibrin sealant) significantly reduces both the incidence and severity of PSAs in rabbit (refer to Chapters 4, 6 and 7) and porcine (refer to Chapter 8) experimental abrasion models. Although many studies, some within this thesis, have utilised the rabbit abrasion model only one pilot study has utilised the porcine abrasion model, therefore further data is required on this second model.

The aims of this study were to:

i. further substantiate PSA prevention with novel fibrin sealant in a definitive porcine abrasion model using quantitative and statistical analyses.
ii. define PSA pathogenesis in a porcine model.
iii. identify possible mechanisms used by novel fibrin sealant to prevent PSA development in a porcine model.

9.2 Materials and Methods

This study consisted of two experimental groups: (i) Controls, and (ii) Human derived novel fibrin sealant treated. Each animal in this study was injured at two sites, stomach and colon (together with apposed peritoneum) with each experimental site randomised to either the control or treated group (Table 9.1). Surgical procedures were as described in Chapter 2, with novel fibrin sealant derived from human whole blood via the Vivostat™ processor. The 100µl Vivostat™ applicator was used to apply the sealant to injury sites, with volumes ranging between 1.2 - 2.0ml with a mean of 1.34±0.07ml. Mean fibrin I concentration was 17.84±0.59mg/ml.

Treatment Group	Number of Animals	Injury Site	Number of Experimental Sites
Control	5	Stomach Colon	5 5 Total : 10
Vivostat™ system human derived fibrin sealant	6	Stomach Colon	6 6 Total: 12

Table 9.1: Study group details.

All animals were recovered for 14 days, with termination and histological procedures as described in Chapter 2. Fixed tissues were uniformly randomly sliced and processed to paraffin wax. A 5μm section was cut from each block and stained with H&E for volumetric analysis using Cavalieri's principal.

9.3 Results

During the course of this study one animal died (allocated to the control group). Cause of death was a strangulated hernia due to suture breakdown. There were no other morbidity or mortalities with any other animal in this study.

9.3.1 Macroscopic Results

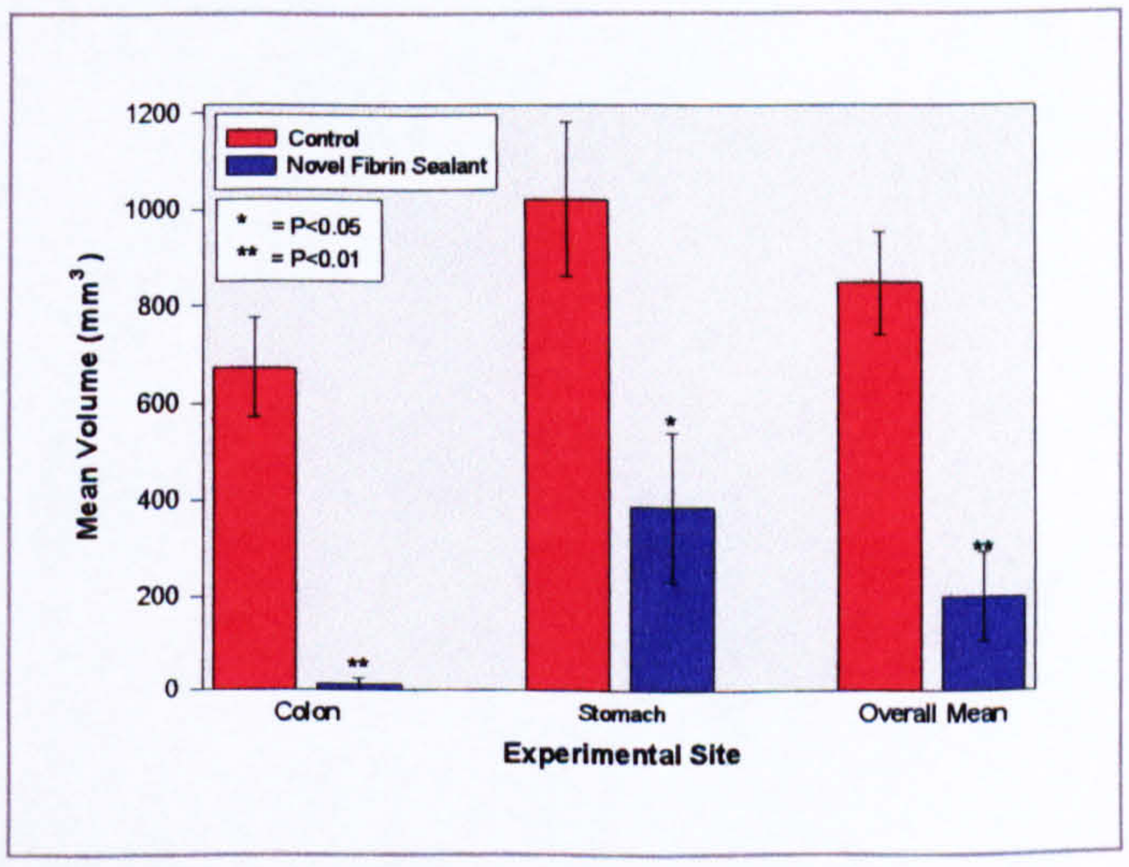
All animals demonstrated PSAs associated with laparotomy, that is, PSAs between the peritoneal suture line and underlying tissues (omentum, colon, liver and spleen). None of these PSAs interfered with any experimental site.

10/10 control sites were fully adhered along their length whereas only 2/12 novel fibrin sealant treated sites were adhered. However 7/12 novel fibrin sealant treated sites, although clear in the centre of the experimental site, showed PSAs on the outside edge(s).

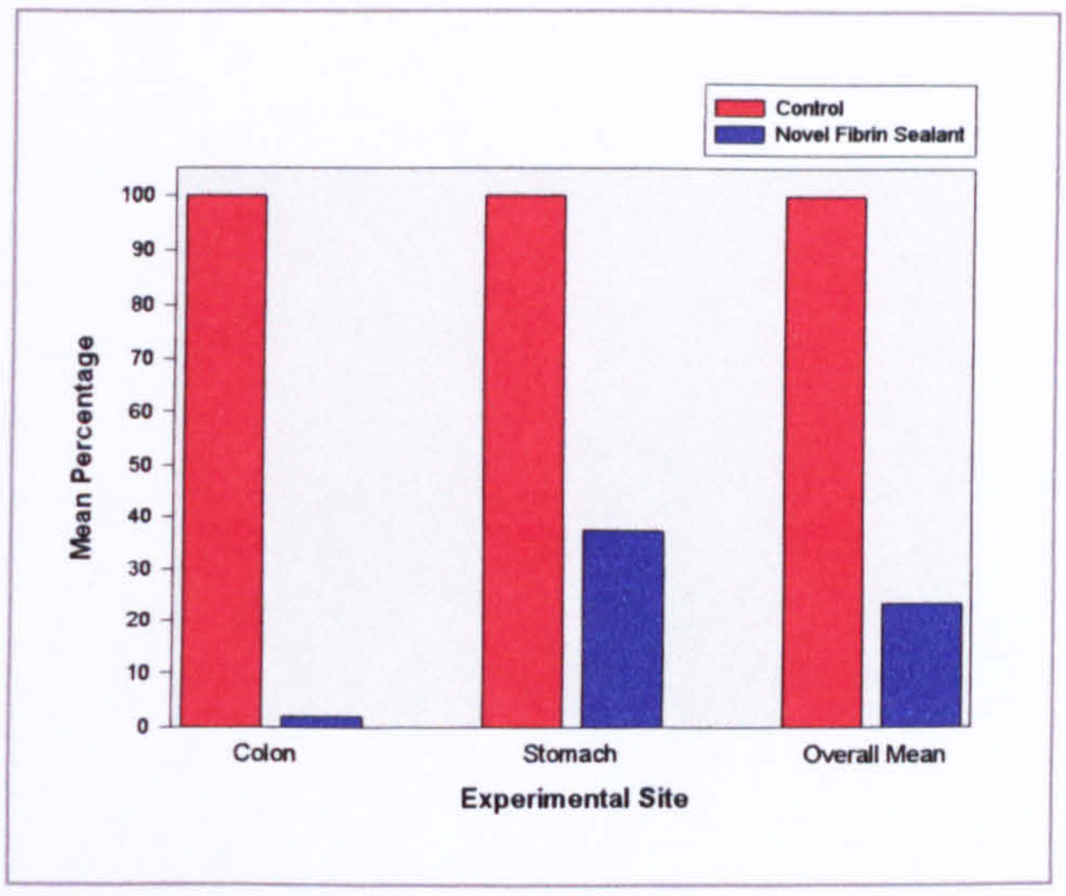
9.3.2 Microscopic Quantitative Analysis

Mean volumes of PSAs at both colon and stomach experimental sites were significantly less ($P>0.01$ and $P>0.05$ respectively) in the novel fibrin sealant group compared to controls (Graph 9.1; Appendix IX Tables 9.1 and 9.2). If it is assumed that the control group represents 100% PSA volume, the incidence of PSAs in the novel fibrin sealant group was 1.92% at colon sites and 37.83% at stomach sites, with an overall incidence of 23.57% (Graph 9.2; Appendix IX Table 9.1).

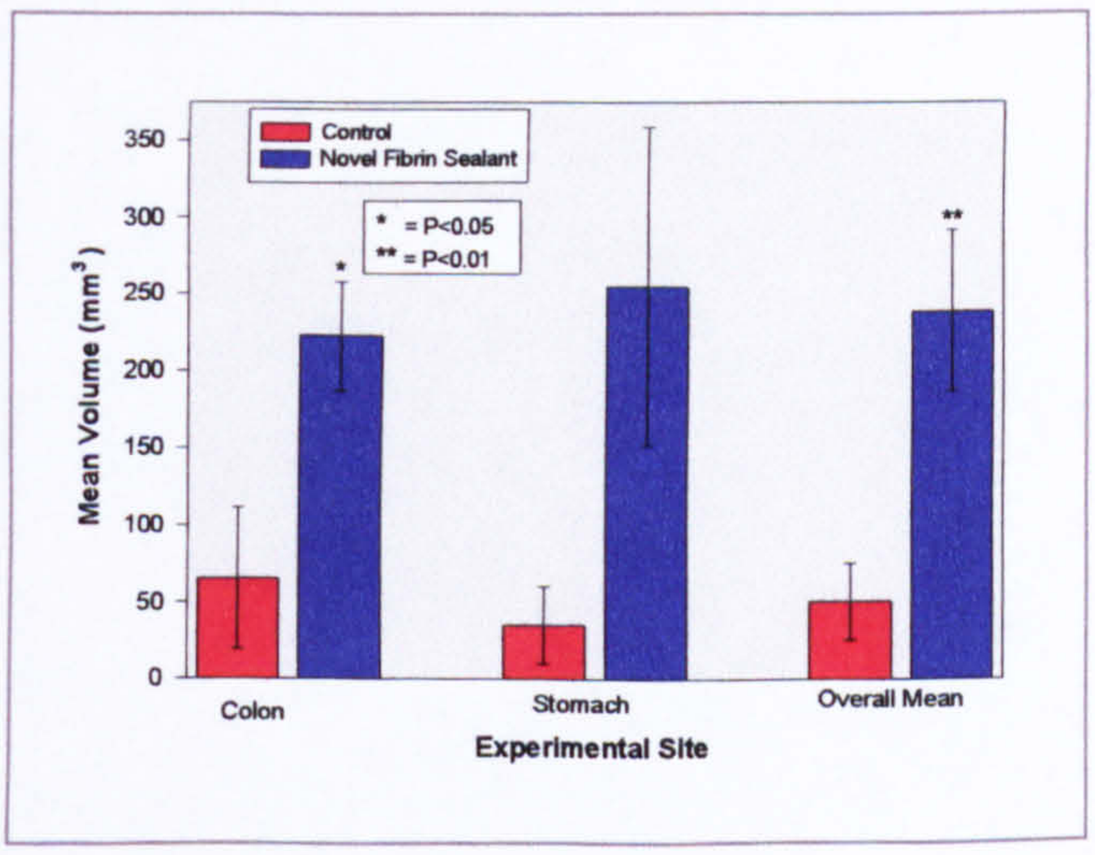
Peritoneal and colon or stomach tissue generation mean volumes were greater in the novel fibrin sealant group compared to controls (Graphs 9.3 and 9.4; Appendix IX Table 9.1). Significantly greater ($P<0.05$) mean volumes were seen at treated colon sites, with regard to both colon and peritoneal tissue generation (Appendix IX Table 9.2) and overall mean peritoneal and colon or stomach tissue generation volumes.



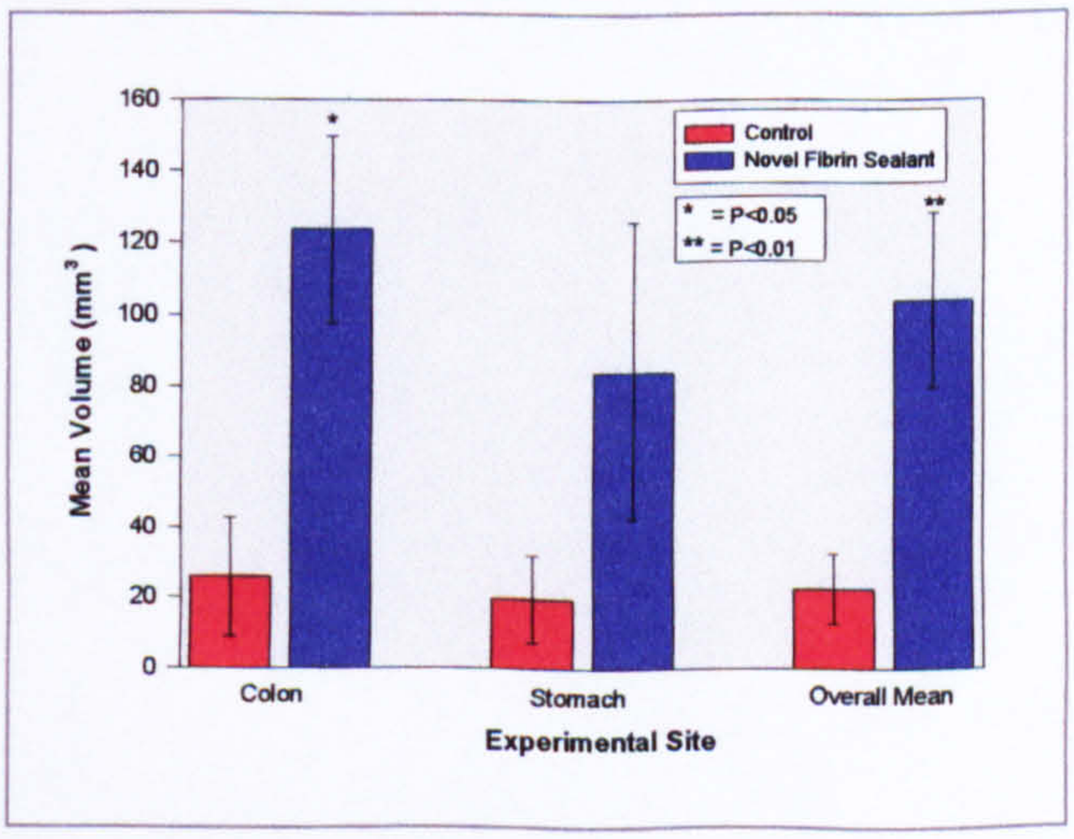
Graph 9.1: Mean PSA volumes
together with SEMs and statistical differences.



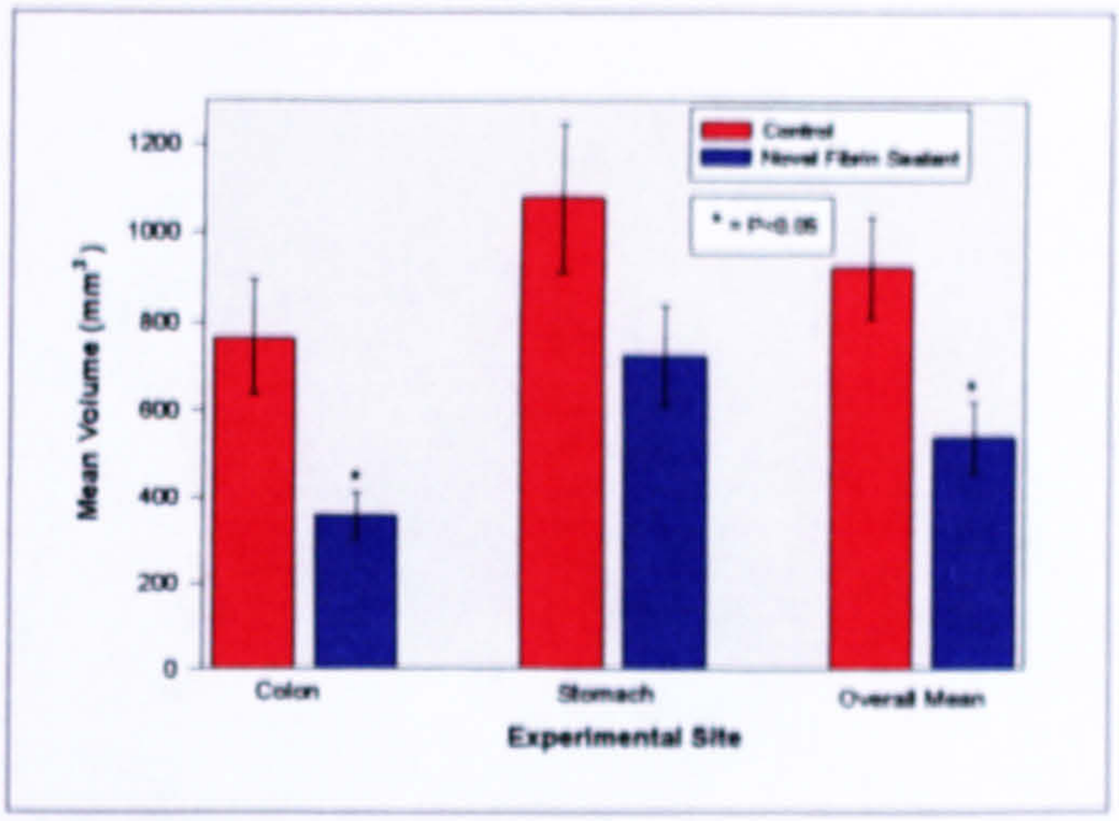
Graph 9.2: Mean PSA percentages.



Graph 9.3: Mean PTG volumes
together with SEMs and statistical differences.



Graph 9.4: Mean colon or stomach tissue generation volumes
together with SEMs and statistical differences.

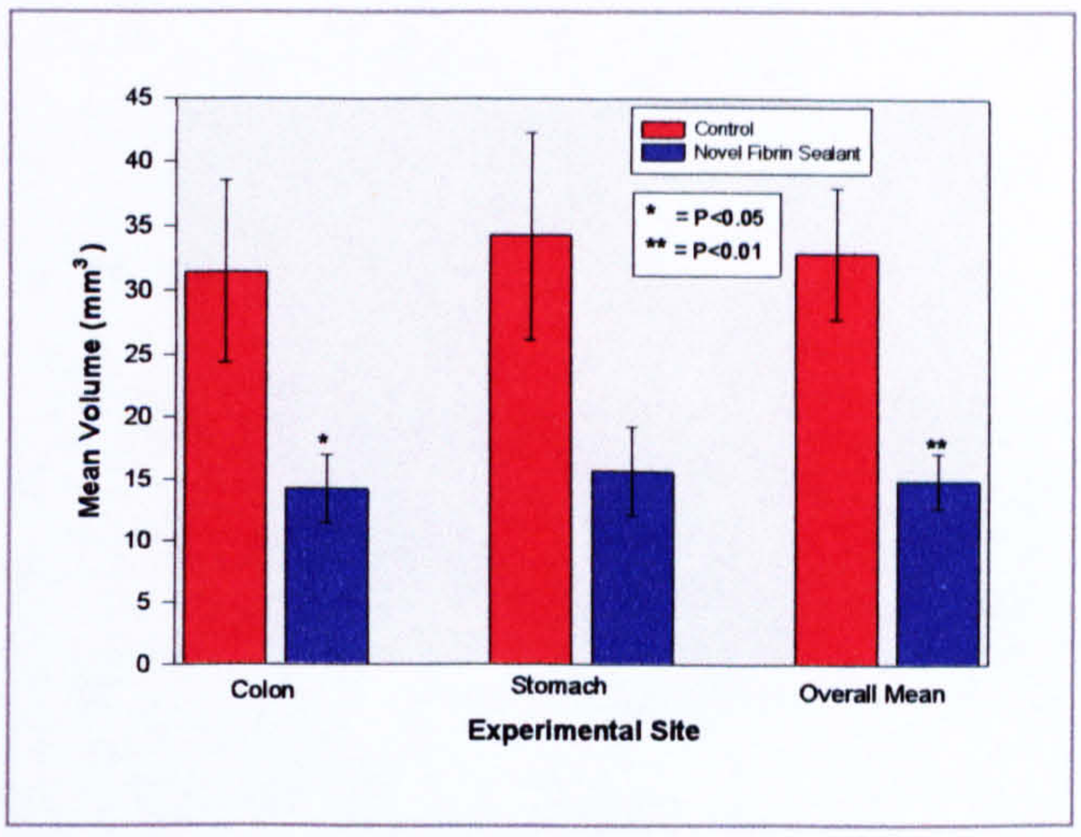


Graph 9.5: Mean TTG volumes
together with SEMs and statistical differences.

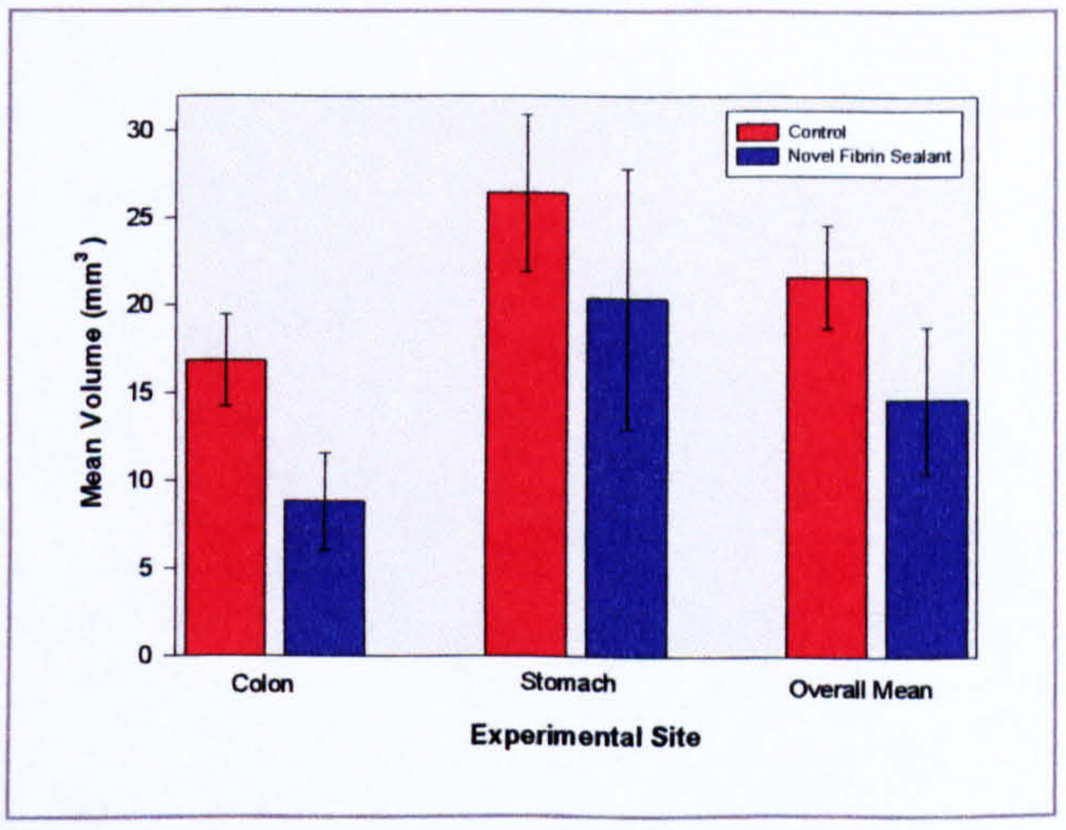
However mean volumes of total tissue generation (PSA and tissue generation volumes) were lower in the novel fibrin sealant group compared to controls (Graph 9.5; Appendix IX Table 9.1). Significantly lower ($P>0.05$) volumes of total tissue generation were found at colon sites and overall means of novel fibrin sealant treated cases compared to controls (Appendix IX Table 9.2).

Experimental sites treated with novel fibrin sealant demonstrated lower mean volumes of peritoneal and colon or stomach damage compared to controls (Graphs 9.6 and 9.7; Appendix IX Table 9.1). Significantly lower mean volumes were present in the peritoneum of colon treated sites ($P>0.05$) and overall peritoneal damage ($P>0.01$) of novel fibrin sealant group compared to controls (Appendix IX Table 9.2).

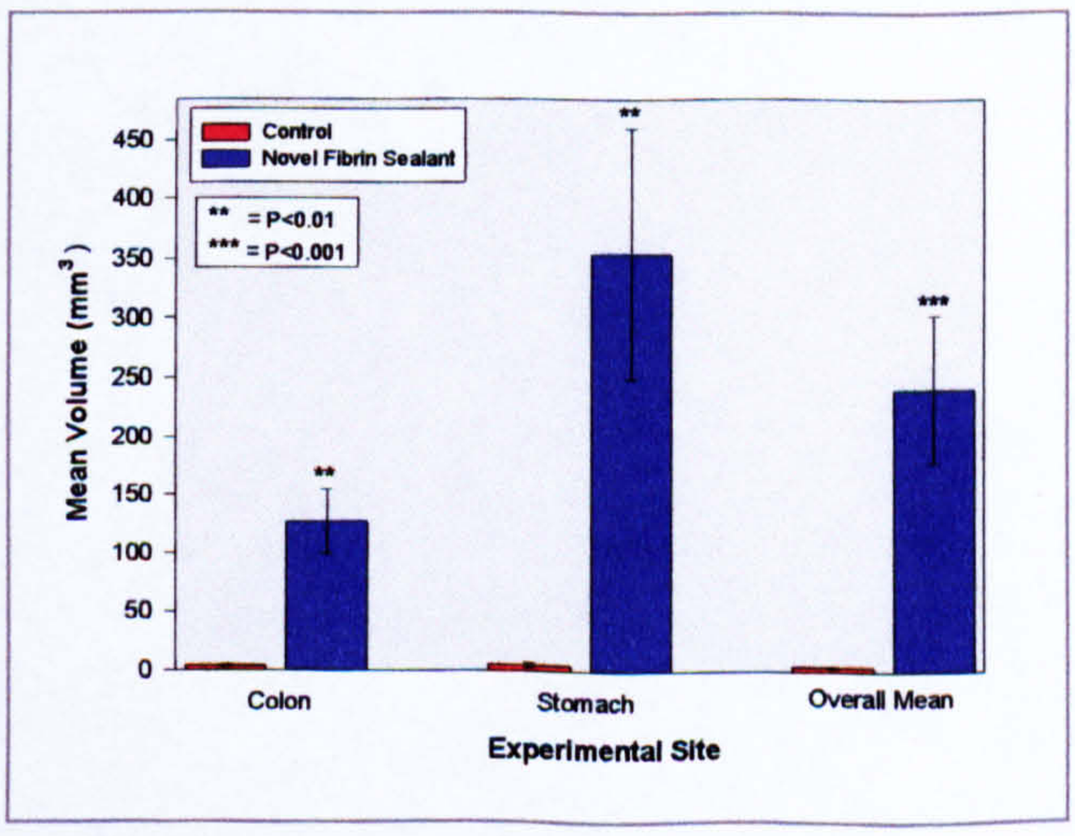
Significantly greater ($P>0.01$) mean volumes of inflammation were present in novel fibrin sealant sites compared to controls at all experimental sites (Graph 9.8; Appendix IX Tables 9.1 and 9.2).



Graph 9.6: Mean peritoneal damage volumes
together with SEMs and statistical differences.



Graph 9.7: Mean colon or stomach damage volumes
together with SEMs.

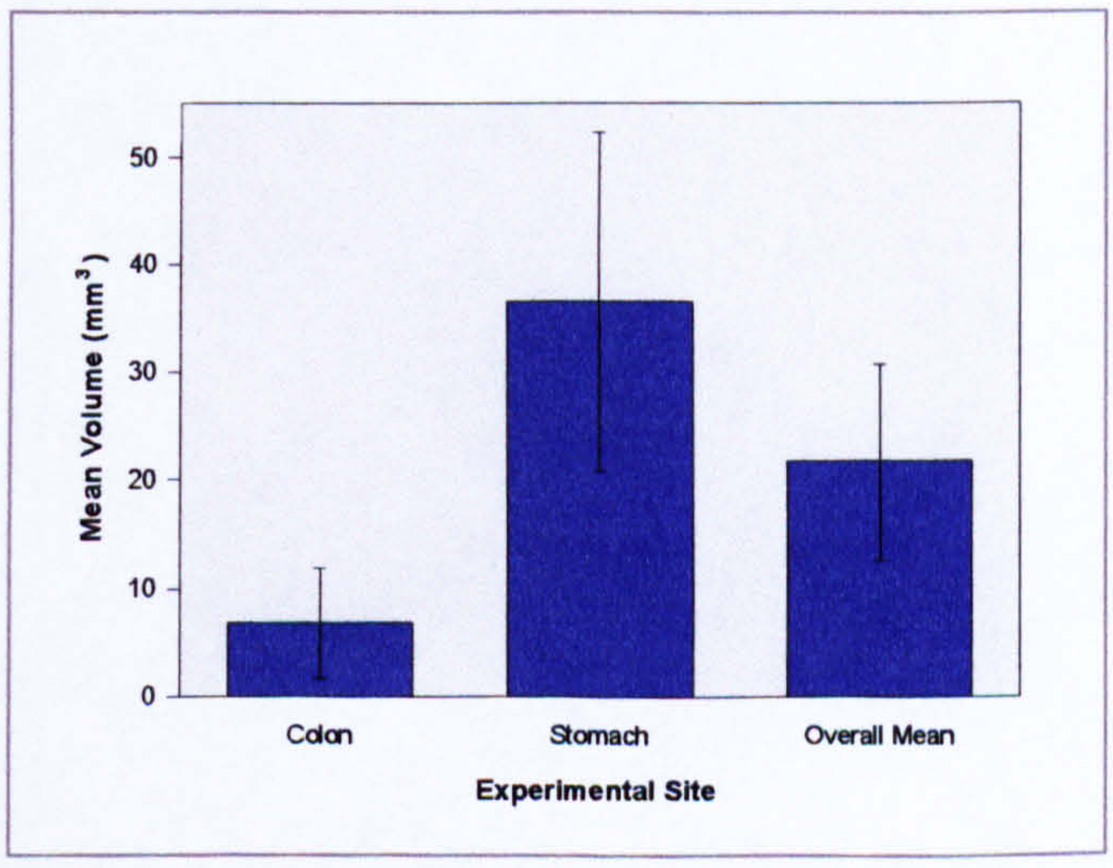


Graph 9.8: Mean local inflammatory reaction volumes
together with SEMs and statistical differences.

33.3% of colon treated sites and 100% of stomach treated sites demonstrated novel fibrin sealant present (Graph 9.9; Appendix IX Table 9.1). Stomach experimental sites demonstrated greater mean volumes of sealant present compared to colon although not significantly ($P>0.05$) (Appendix IX Table 9.3).

There were no significant differences ($P>0.05$) between colon and stomach sites in the control group for tissue zones analysed (Appendix IX Table 9.3). However significant differences were present in the novel fibrin sealant group: significantly greater mean volumes of PSA ($P<0.01$), total tissue generation ($P<0.05$) and inflammation ($P<0.05$) were present at the stomach compared to colon site.

The overall coefficient of error for this study was 13%.



Graph 9.9: Mean novel fibrin sealant remaining volumes together with SEMs.

9.4 Discussion

There have been very few studies investigating PSAs which have utilised large experimental models such as the pig, due primarily to their increased cost and with smaller models being acceptable for most PSA work. Those studies which have used porcine models have found that pigs generated PSAs in response to injury or trauma (Fowler *et al.*, 1994; Christoforoni *et al.*, 1996; Reissman *et al.*, 1996) and have shown

some success using potential PSA preventative agents.

The porcine model used in this study produced 100% PSA incidence in response to the PSA inducing abrasion process with no clinical morbidity. Hence this porcine peritoneal to colon or stomach abrasion model is a valid and definitive PSA experimental model. When used in conjunction with quantitative analysis it is an ideal large experimental model for investigating both PSA development and prevention with PSA preventative agents.

Treatment with novel fibrin sealant significantly reduced PSA volume with a 98% reduction at colon sites and 62% at stomach sites and an overall reduction of 76%. With regard to incidence of PSAs, there was a 83% reduction at colon sites, no reduction at stomach sites but a 42% reduction overall. The reason that incidence reduction of PSAs does not follow the same pattern as volume reduction is that at many treated stomach sites the central area was completely clear of PSAs however PSAs were present on outside edges of the experimental sites. Hence in areas were novel fibrin sealant was present there were no PSAs but in areas which were not fully covered or were bordering non-covered areas PSAs developed. Reasons for incomplete coverage could be due to poor sealant application or more probably the elasticity of the stomach. However it is clearly evident that novel fibrin sealant reduced both incidence and severity of PSAs in this porcine model.

The response to abrasion injury at both peritoneal and colon or stomach treated experimental sites was injury tissue which was generally discretely located on either surface, rather than joining the two. Peritoneal and colon or stomach tissue generation volumes were greater in treated cases compared to controls. However, when comparing total tissue generated, controls showed greater mean volumes compared to treated cases. Thus, in addition to affecting the location of injury tissue, treatment with novel fibrin sealant decreased the volume of injury tissue generated. This is most probably due to the lower damage volumes seen at 14 days in the novel fibrin sealant group.

Since all animals underwent the same injury process, volumes of the original abrasion injury will have been similar for control and treated groups. However, by 14 days, lower damage volumes were seen in the treated group indicating that they either had decreased subsequent damage (due to necrosis of surrounding and underlying tissue) or that damage was repaired at a faster rate compared to controls. Since there is a direct relationship between volume of damage and tissue generated in response to that damage (Ellis, 1962; Belzer, 1967; Chapter 3) the first of these hypotheses is the most probable. Treatment with novel fibrin sealant limits surrounding and underlying tissue damage and subsequently tissue generated in response to damage.

Significantly greater volumes of inflammation were seen in novel fibrin sealant treated cases compared to controls. For this study peritoneal reaction mounted in response to injury and cell mediated response to novel fibrin sealant was termed inflammation. Increased inflammation seen with treated cases was primarily due to the immune response mounted to the presence of human protein within the pig peritoneum. This is highlighted by greater volumes of inflammation at stomach experimental site at which there are greater volumes of fibrin sealant remaining. Reasons for the greater residency of novel fibrin sealant at stomach compared to colon experimental sites included potential differences in fibrinolytic capacities (Marshall, 1985) and motility of these tissues.

Conclusions

i. This porcine peritoneum to colon or stomach abrasion model is a valid definitive model for investigating PSA development and prevention.
ii. Novel fibrin sealant significantly reduced both incidence and severity of PSAs.
iii. Novel fibrin sealant demonstrated a protective effect against necrosis of surrounding and underlying tissues with reduced injury tissue the final outcome.
iv. Human novel fibrin sealant produced an inflammatory response in the pig peritoneum.
v. The residency time of novel fibrin sealant was influenced by its location within the peritoneal cavity.

10.0 Discussion

The formation of adhesions following surgical therapy is almost inevitable. PSA presence is directly related to number of operations (Weibel & Majno, 1973; Luijendijk *et al.,* 1996) and there is a 93% incidence following major abdominal surgery (Menzies and Ellis, 1990; Duron *et al.,* 1993). In today's medical climate, with many patients undergoing multiple surgery therapies, PSAs are more pertinent than ever. Not only can PSAs result in the need for re-operation due to associated pathogenesis or failed surgical therapy but they also make re-operation difficult. This is especially problematic in cardiac surgery where the formation of PSAs ultimately limits the number of operations which can be attempted.

The actual mechanics of surgery present ideal situations for PSA development: tissue injury and/or ischaemia, bleeding, introduction of foreign materials, potential presentation of infectious agents and reduced fibrinolytic capacity. All of these factors have been identified as causes of PSAs with authors choosing their preferences (refer to Chapter 1). It is most probably a combination of these factors which promote PSA formation in all types of surgical procedures.

In order to gain a comprehensive clinical understanding of PSA formation, maturation and finally prevention, valid experimental models are required. An ideal PSA experimental model is one in which PSAs are induced in 100% of cases via standardised and reproducible techniques which have good clinical representation. Unfortunately this has not been the trend with regard to most PSA models. Previous experimental models have shown variations in animal species (rat, rabbit, pig, dog, monkey, calf, chicken and human), induction methods (rubbing of caecal surface, chemical trauma, crushing caecal wall, mesentric division, polythene/ omental wrap around devascularised intestine and liver lobe scraping with scapula blade), severity of techniques (rubbing, crushing, and scalpel scraping), site of PSA induction (caecum, intestine, liver and uterine horn) and end point (incidence, length of adhesion attachment and type of adhesion) (Appendix X Table 10.1). With such divergent models, collation of published data, which may be morphological, cellular or biochemical, is very difficult. Abrasion models, rabbit and

porcine, utilised in the studies contained within this thesis eliminate problems encountered previously. The PSA induction technique was standardised with regard to the area of tissue injured and the amount of abrasion procured and was designed to be a severe PSA inducer. Although for the purpose of investigating PSA formation through normal surgical trauma these models may be extreme, they were also designed with a view to test potential PSA preventative agents where a potent PSA inciter is a necessity.

Another major benefit of the data contained within this thesis compared to most previously published PSA data is that it was quantitatively microscopically analysed using strict unbiased techniques. Qualitative analyses have been predominant in this field of science and have the innate problems of inaccuracy, imprecision and potential for bias. These are eliminated with stereological techniques as long as basic principles are observed (refer to Chapter 2). Differences between qualitative macroscopical data and quantitative microscopical data are highlighted in the results contained within this thesis (Table 10.1)

Study	Chapter Number	Percentage Incidence of PSAs		% PSA Volume
		Macroscopic Analysis	Microscopic Analysis	
Pathogenesis of PSAs	3	90	85	100
Prevention of PSAs	4	2	2	-
Application Comparison	6	Controls: 100 Treated: 21	Controls: 100 Treated: 63	Controls: 100 Treated: 29
Fibrin Sealant Comparison	7	Controls: 100 Treated: 71	Controls: 100 Treated: 80	Controls: 100 Treated: 105
Pig PSA Pilot Study	8	Controls: 100 Treated: 0 - 42	Controls: 100 Treated: 62	Controls: 100 Treated: 22
Pig PSA Main Study	9	Controls: 100 Treated: ? 58	Controls: 100 Treated: 58	Controls: 100 Treated: 24

Table 10.1: Percentage incidence of PSAs.

Although both incidence and volume of PSAs for controls groups do not vary this is certainly not the case for treated groups. In general, macroscopic incidence of PSAs is less than microscopic due to the more strict and precise detection by microscopical methods. Thus PSA reduction has a trend to be overestimated by macroscopical observations which is highly significant for determining the efficacy of potential PSA preventative agents. In order to gain a comprehensive idea of PSA presence and

reduction, the severity of PSA, determined by accurate microscopically analyses, is actually a more precise and valid method of evaluating potential anti-PSA agents. There is a potential for PSAs to be disturbed during processing to microscopical material, however, the results of these studies demonstrate that, with normal careful tissue handling, this should not occur after 8 hours post injury. Of course such microscopical analyses are only normally possible for pre-clinical studies but if such strict assessments can be made at this stage good clinical results should be the reward.

Accurate knowledge concerning the formation and maturation of PSAs is essential for limitation of PSAs through surgical practices, development of effective preventative agents and also for a degree of acceptance by surgeons. Limitation of PSA formation through meticulous surgical techniques is well accepted, with methods such as strict asepsis, minimal gentle handling of tissues, avoidance of foreign material contamination, to name just a few (refer to Chapter 1). However studies by Scott-Coombs et al (1993) and Holmdahl & Risberg (1997) highlight that maximum surgical care is not routinely practised by surgeons, leading to the formation of avoidable PSAs with the main culprits being the use of starch gloves and suturing the peritoneum. The studies contained within this thesis also demonstrated the importance of damage, especially underlying and non-visible, on the formation of PSAs. Therefore all limitation techniques need to be fully implemented to help combat PSAs.

Although significant effort and resources have been directed against finding potential PSA preventative agents there is to date no such product available. There has been a typical trend seen with these agents: initial enthusiasm followed by more comprehensive experiments which result in less encouraging data and ultimately abandonment, with very few agents ever reaching clinical trials (Ellis, 1982). The main reason for the high failure rate amongst these products is due to lack of knowledge concerning mechanisms governing PSA development. Potential preventative agents have been mainly targeted towards preventing PSAs primarily by separating surfaces but also by lysis of formed PSAs by chemical or mechanical means, with little consideration of subsequent repercussions. With information on the dynamics of PSA development, areas suitable for targeting agents can be deduced together with the optimal timing for prevention and

the physiological effects of such prevention at that stage of PSA development.

Due to the pathogenesis and problems associated with PSAs they are generally seen as sinister, a result of wound healing "gone wrong"/excessive wound healing. The results of this study demonstrate the formation and maturation of PSAs to be a highly regulated complex process which is the bodies natural defence to injury and inflammation. There are other authors who have expressed positive views on PSAs: Ellis (1962) described adhesions as "vascular grafts" preventing necrosis, gangrene or perforation; Myllarniemi & Karpinen (1968) stated that preventing adhesions may hamper recovery, promoting infection leading to gangrene and necrosis; Deaver (1923) described adhesions as ladders for phagocytic cells to reach injured tissues to limit infection. Hence the formation of PSAs following surgical therapy, inflammation and/or infection has the benefit of acting as a pathway for cells, nutrients and chemical mediators to reach damaged tissues quickly. Therefore PSAs should gain a greater respect within the surgical community.

Since PSAs only lead to pathogeneses in a minority of cases, some authors (for example: Ellis) feel that PSAs should not been seen as sinister and that they should received less attention. However in an era where many patients undergo multiple surgical therapies and economic awareness is at its greatest the problems associated with PSA formation overshadow any advantages of their presence.

This is the first study to simultaneously analyse PSA development with regard to physiological, structural, cellular (local and systemic) and biochemical mechanisms. The dynamics of PSA development are shown in Figure 10.1 demonstrating the formation and maturation of PSAs to be a complex, multi-system and controlled process. Comparison with adhesion-free healing (Section 1.1.6.3) and PSA development (Section 1.1.6.5) show compatible results with regard to mechanisms involved.

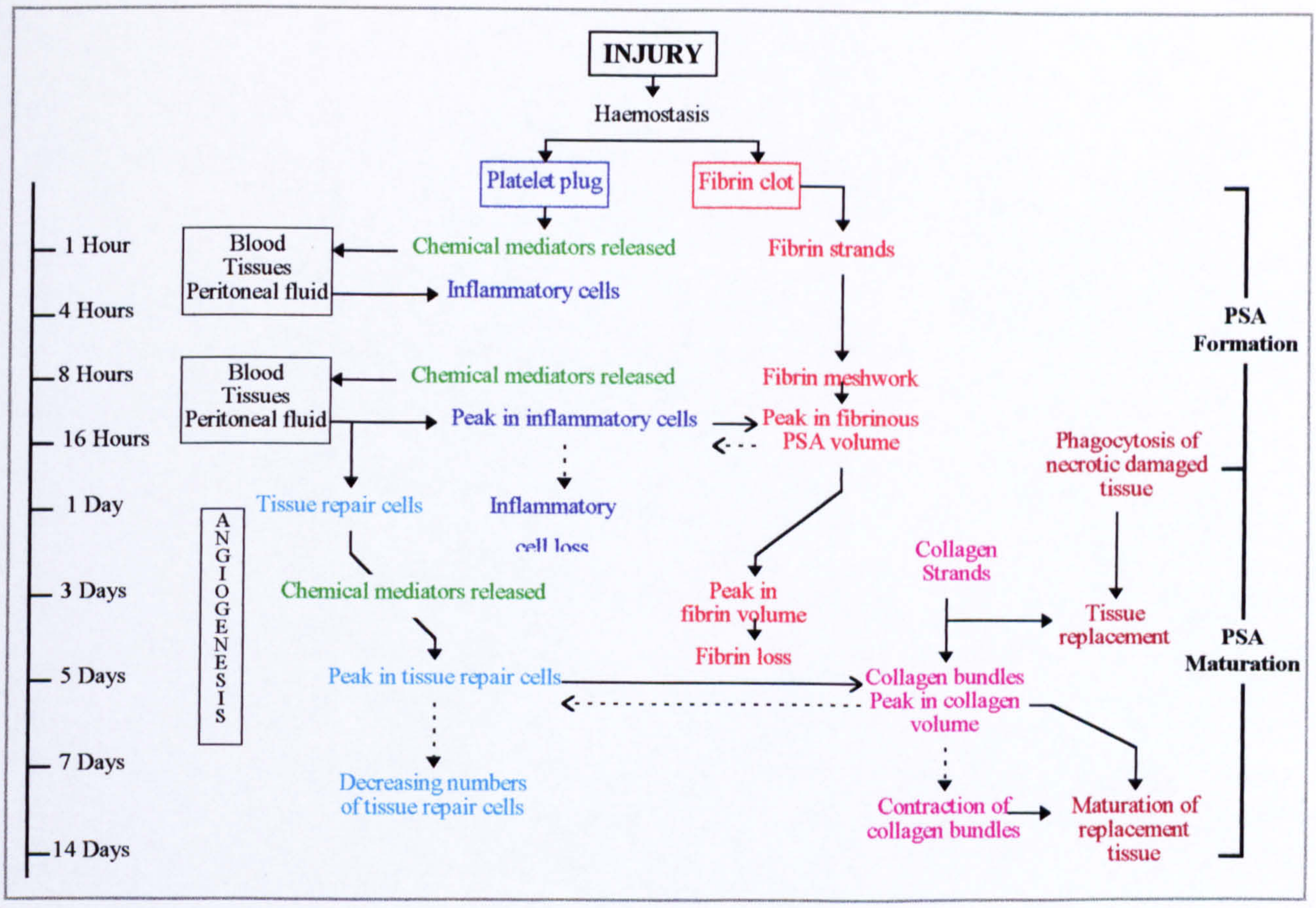


Figure 10.1: PSA development.

It would appear that the only reason that PSA development occurs as an alternative to adhesion-free healing is due to fibrin exudated from damaged surfaces coming in contact with another surface, which has some degree of damage, visible at the macroscopic level or not, which provides an adherent interface. It is once PSAs have formed that their presence propagates their existence, deviating slightly from adhesion-free healing. If we assume that tissue generated from the peritoneum and uterine horn which does not form PSAs is typical of adhesion-free healing then the dynamics of their formation are dependent upon volume of damage and regeneration of repair tissue to replace removed necrotic and non-viable tissue. The effects of the inflammatory response are not noted on tissue generation but have the indirect effect on their volume by involvement in the clearing of damaged tissue and therefore the resulting tissue generation. However this is not the case with PSA development. The inflammatory response demonstrated a direct effect on the volume of fibrinous PSAs and, of course, tissue repair showed a similar effect on fibrous PSA volume. Hence adhesion-free tissue generation is solely concerned with replacement of damaged tissue, that is, wound

healing, whereas PSA development is associated with the inflammatory response and to a degree wound healing. Thus the inflammatory response is very important for PSA formation and as such is a potential target for PSA prevention.

The overall incidence of PSAs during the PSA development study was 85%. The injury used in these studies was a severe PSA inducer and hence all cases probably developed primary fibrinous PSAs. The actual incidence for fibrinous PSAs (8 hours to 3 days) was 97%, with only one 3 day experimental site lacking PSAs. Whereas the incidence for fibrous PSAs (5 to 42 days) was 75%. Hence some animals which, in all probability, would have developed fibrinous PSAs, lysed this primary PSA evading the formation of established fibrous PSAs. From the data obtained during these studies we know that from 3 days onwards there is a rapid decrease in fibrin volume, that is, loss of fibrinous PSA volume and at the same time collagen synthesis is just beginning in earnest. Hence there is a transition period in which fibrinous PSAs are being broken down and fibrous PSA being established and it was at this time that the first drop in PSA incidence was seen. This is another potential target for PSA prevention.

Previous studies have demonstrated a significant drop to complete removal of fibrinolytic activity following trauma for at least the first 24 hours following trauma (Ryan *et al.*, 1973; Raftery, 1981; Scott-Coombs *et al.*, 1995). This will naturally promote the formation of fibrinous PSAs. However once this activity returns and, in animals which either have greater fibrinolytic capacities or have a more rapid return of fibrinolytic activity, lysis of fibrinous PSA occurs at this stage. In animals in which this does not happen, fibrin lysis occurs from 3 days onwards however the linkage already contains collagen and thus an established PSA develops.

Within 14 days post injury, PSA development and maturation was near completion. The physiological parameters were characteristic of an established PSA, with cellular content limited primarily to fibroblasts trapped within the contracting collagen matrix.

With regard to PSA prevention the ideal solution is to prevent fibrinous linkages from forming, which is probably achieved most easily by a physical barrier placed at the time

of surgery. Increased fibrinolytic activity could be promoted, although this may carry of risk of interfering with haemostasis. An alternative would be to break the fibrinous PSA that is formed during the first 1 to 3 days following injury. This could be achieved via fibrinolytic agents although care would need to be taken to not disrupt wound healing, or via mechanical methods although this would carry the risk of inducing further PSA development. What ever agent is chosen it should not induce an inflammatory response, which is directly associated with PSA formation and promotion. With regard to the residency time of a potential PSA agent it should prevent fibrinous PSAs and in turn fibrous PSAs. We know from these studies that fibrin deposition during PSA development occurs for at least the first 3 days post injury, so this is the minimal residency time for PSA prevention agents.

Comparison of data obtained from PSA prevention with novel fibrin sealant and previous studies investigating adhesion-free healing (Section 1.1.6.3) and PSA development (Section 1.1.6.5) showed similar dynamics with regard to structural, cellular and biochemical processes (Figure 10.2). Hence treatment with novel fibrin sealant does not change or interfere with normal wound healing mechanisms. Since the only other study to quantitatively analyses these dynamics was the PSA development study then only direct comparisons with this study can be made.

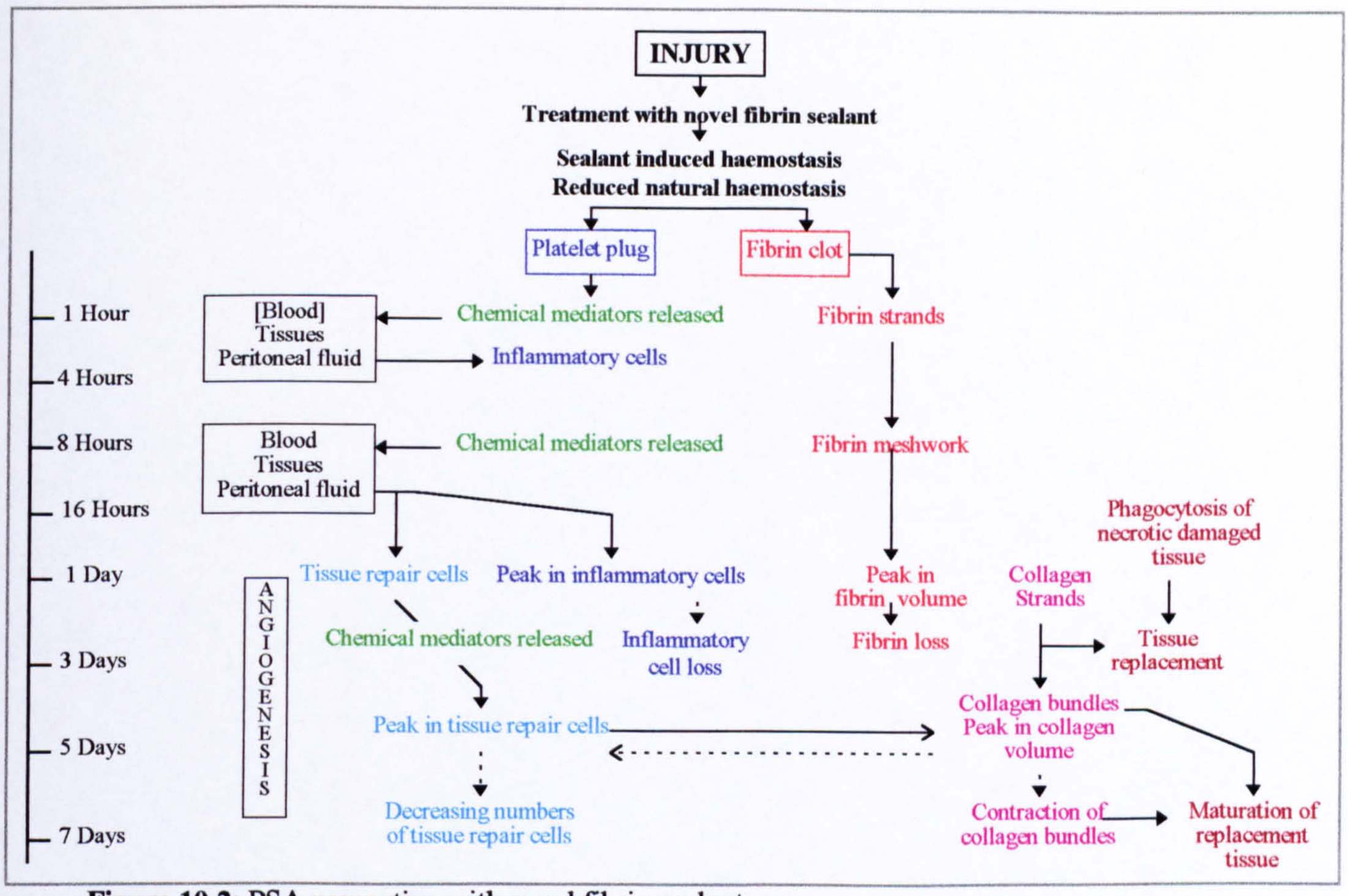


Figure 10.2: PSA prevention with novel fibrin sealant.

Although the same pattern was seen for PSA development, treatment with novel fibrin sealant had the effects of: (i) decreasing volume of necrosis and underlying tissue damage; (ii) decreasing the volume of tissue generated in response to that injury; (iii) decreasing the volume of reaction within peritoneal tissue; (iv) increasing the rate of structural changes; (v) decreasing the magnitude of inflammatory and tissue repair cell presence, (vi) decreasing systemic cellular number changes; and (vii) decreasing biochemical mediators (TGF-β). Although all aspects of wound healing have been altered, these alterations probably result from changes at the first stage of wound healing, haemostasis. Fibrin sealants have been developed primarily as haemostatic agents, with their potential to reduced PSA formation discovered by accident during second-look procedures. Since fibrin sealants modulate haemostasis, the bodies natural haemostatic practices will be reduced. This will in turn result in a lesser presence of platelets and natural fibrin at the injury site. Platelets are very important in the initial stages of wound healing, with their degranulation leading to vasoconstriction, the formation of a platelet plug, fibrin deposition and chemotaxis of inflammatory cells.

With reduced platelet number the amount of chemical mediators released will also be reduced, resulting in lower numbers of inflammatory cells, fibrin lay down and in turn lower numbers of tissue repair cells and fibrotic tissue. The net result of this is accelerated, PSA-free wound healing.

It could however be argued that novel fibrin sealant is bio-active, that is, healing processes are affected by chemical mediators released from the sealant when *in vivo*. Further biochemical analyses are required to clarify this situation.

The importance of the peritoneal environment should not be overlooked. Previous studies have demonstrated that peritoneal fluid is a medium for cellular (Eskeland, 1966.1; Eskeland, 1966.2; Raftery, 1973.L; Shimanuki *et al.,* 1986) and biochemical interactions (Rong *et al.,* 1990; Ford *et al.,* 1990) which may modulate tissue remodelling (Ford *et al.,* 1990). The preliminary results of TGF-β concentrations within peritoneal fluid of control and novel fibrin sealant treated cases lends support for this hypothesis. Hence isolation of injured tissue from peritoneal fluid may be sufficient to cause the changes seen. Support for this comes from the similar dynamics of PSA formation and tissue generation from treated cases both of which are isolated from the immediate peritoneal environment. Inflammatory cells were associated with fibrinous tissue deposition and tissue repair cells with fibrous tissue. Whereas in tissue generation of control cases, which had direct contact with the peritoneal environment, fibrinous tissue formation appeared to be independent of inflammatory cell interactions. This isolation theory has also been identified by Sheppard *et al.* (1993) who stated that fibrin sealant acts as a physical barrier as well by isolating the healing wound and its inflammatory mediating factors from the rest of the site.

Although biochemical modulation of wound healing by novel fibrin sealant can not be ruled out at the present time, it is most probable that PSA prevention is primarily due to the sealant inducing artificial haemostasis and acting as a physical barrier, separating injured surfaces from themselves and other surfaces and the peritoneal environment.

These studies have shown that PSA development has a close relationship with the

inflammatory response, therefore, ideally, an anti-PSA agent should not induce any additional inflammatory reaction. However, in these studies investigating human derived novel fibrin sealant in a rabbit experimental model, an inflammatory response was mounted against the sealant. This in theory should have reduced PSA prevention in these cases however this does not appear to be the case. The reaction to the sealant present was highly concentrated around the sealant and did not really impinge on the tissues themselves and this the reason why this local immune mediated foreign body reaction was not overly detrimental.

From spraying of the sealant to 1 hour *in vivo* there was a 89% decrease in volume of novel fibrin sealant. Since fibrinolytic activity is almost totally eradicated for at least the first 24 hours following surgery (Ryan *et al.,* 1973; Raftery, 1981; Scott-Coombs *et al.,* 1995), lysis of the fibrin sealant is highly improbable. Therefore reasons for this dramatic decrease are due to loss by aerosol during the spraying process, over spraying of tissues due to spray pressure and fluid loss from the sealant. This dehydration was continued until 4 hours post application. Hence the volume of sealant at 1 to 4 hours post application was the volume of fibrin present *in vivo* available to prevent PSAs. From 4 hours to 5 days the volume of fibrin present remained relatively constant, with sealant joining surfaces for up to 3 days post injury and covering at least one site up to 5 days in all cases. Sealant was present at all experimental sites at 7 days, 63% of cases at 14 days, 50% of cases at 28 days and absent in all cases at 42 days. Therefore once *in vivo* novel fibrin sealant is very stable during the first 5 days following application, that is, during the generation of fibrinous tissue generation and PSAs and conversion to fibrous tissues. This is a critical time period for PSA development and the stability of preventative agents during these times is very important. Biodegradation of the product is also important and novel fibrin sealant can be seen to gradually decrease from 14 to 28 days and be completely absent by 42 days.

Comparison of PSA reduction with novel fibrin sealant in the studies utilising the rabbit uterine horn abrasion model included in this thesis demonstrated a mean reduction in PSA volume of 79.5% (Table 10.2). Whether novel fibrin sealant was bench or device prepared, applied using the Vivostat™ applicator spray (100µl or 300µl), needle or

UNES gun, significant reduction of PSAs was seen compared to controls (Table 10.3). Differences in PSA reduction efficiency are due primarily, to application methods used. The Vivostat™ spray applicator is the method of choice for novel fibrin sealant and this delivery system has demonstrated a mean PSA reduction of 87%. During the development of Vivostat™ novel fibrin sealant technology two spray systems have been used in recent years, the 300µl spray which was then replaced by the 100µl spray. The 300µl spray (utilised in Application Comparison (Chapter 6) and Fibrin Sealant Comparison (Chapter 7) studies) demonstrated a mean PSA reduction of 81% compared to 99% for the 100µl spray (PSA Prevention Study). Hence this second generation 100µl spray is more effective for the application of novel fibrin sealant for PSA prevention. This highlights the importance of the method of sealant application for prevention of PSAs. In order to achieve complete PSA prevention complete coverage of damaged tissue is essential and this is only achieved via precise application of fibrin sealant by an appropriate application system.

Study	Chapter Number	Preparation Method	Application Method	Percentage Reduction in PSAs
Application Comparison	6	Device	Spray (300µl)	77
		Device	Needle	67
		Device	UNES Gun	56
			UNES Gun	93
Fibrin Sealant Comparison	7	Device	Spray (300µl)	85
PSA Prevention	4	Device	Spray (100µl)	99

Table 10.2: Percentage reduction in PSA volume by novel fibrin sealant in rabbit uterine horn abrasion model studies.

In three different studies, treatment with novel fibrin sealant in an experimental rabbit uterine horn abrasion model consistently significantly reduced volume of PSAs, total tissue generation (TTG), damage and peritoneal reaction and significantly increased volume of cell concentration (Table 10.3). Considering differences in Vivostat™ spray applicators (300µl or 100µl) these studies have yielded similar results. Hence treatment with novel fibrin sealant produces consistent and reproducible effects on wound healing in a definitive rabbit uterine horn model.

Tissue Zone	Study			Overall
	Application Comparison (Chapter 6)	Fibrin Sealant Comparison (Chapter 7)	PSA Prevention (Chapter 4)	
PSA	↓***	↓****	↓*/***	↓
PTG	↑***	↑	↓*/***	↑/↓
UTG	↑	↓	↓*/***	↓/↑
TTG	←→	↓*	↓*/****	↓
Peritoneal damage	↓	↓**	↓*/** (5 Days +)	↓
Uterine horn damage	↓*	↓****	↓*** (3/5 Days +)	↓
Peritoneal reaction	←→	↓**	↓***/****	↓
Peritoneal cell concentration	↑****	↑****	↑*/*** (5 Days +)	↑

Table 10.3: Tissue zone volumes from novel fibrin sealant rabbit uterine horn abrasion studies (Vivostat™ spray only) compared to controls.

Note: ↓ = decreasing volume; ↑ = increasing volume.

* = P<0.05; ** = P<0.01; *** = P<0.001; **** = P<0.0001.

■ = significant difference/expected significant difference.

*/** = minimum significant difference/maximum significant difference.

In the second animal species investigated, porcine abrasion model, reduction of PSAs with human blood derived novel fibrin sealant ranged from 59 to 76% with a mean of 67.5% whereas with porcine blood derived novel fibrin sealant PSA reduction was 99%. Similar results with regard to tissue zone volumes of treated compared to control cases were found (Table 10.4). Treatment with novel fibrin sealant significantly reduced volumes of PSAs, TTG and damage and significantly increased volume of inflammation. Differences between efficacy of PSA prevention between these two experimental models could be due to species differences however is more likely to be due to the different experimental sites investigated. This will be discussed shortly.

Tissue Zone	Study			Overall
	Pilot (Chapter 8)		Main (Chapter 9)	
	Porcine Sealant	Human Sealant	Human Sealant	
PSA	↓↓	↓	↓**	↓
PTG	-	-	↑**	↑
Colon or stomach TG	-	-	↑**	↑
TTG	-	-	↓*	↓
Peritoneal damage	-	-	↓**	↓
Colon or stomach damage	-	-	↓	↓
Inflammation	←→	↑↑	↑***	↑ (Human)

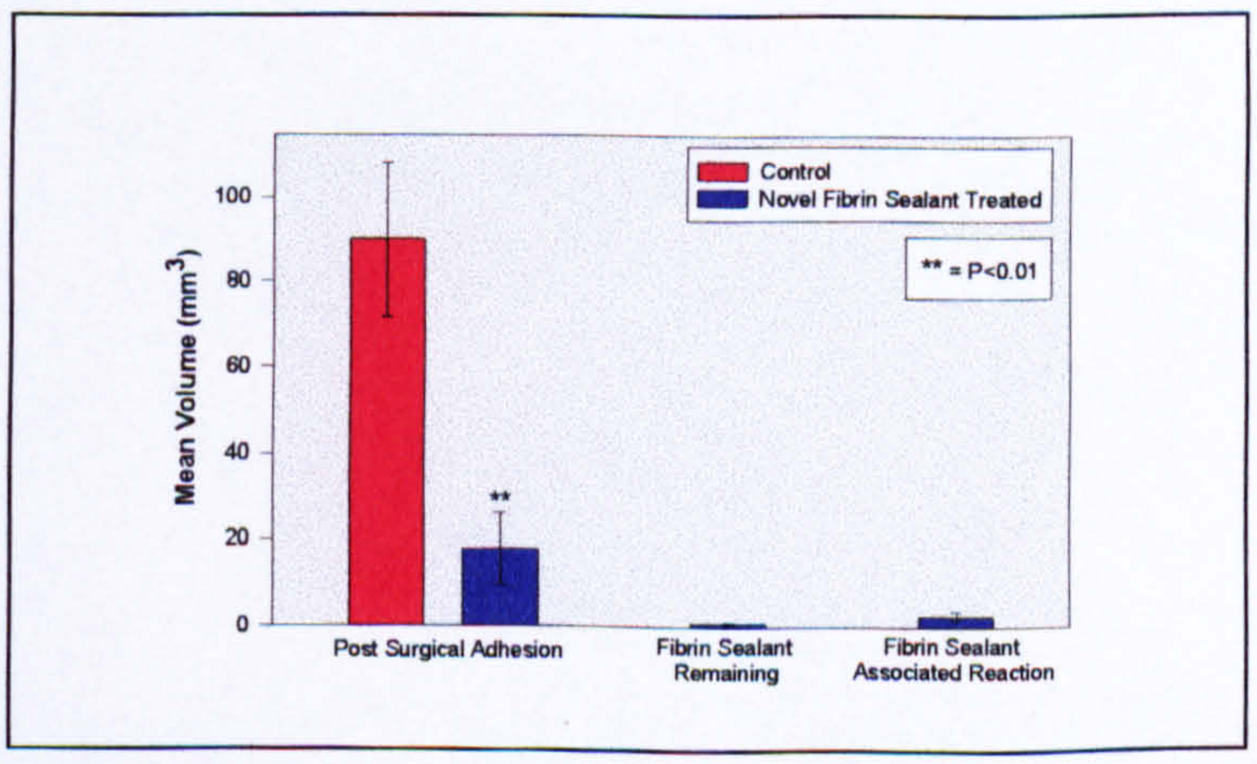
Table 10.4: Tissue zone volumes from novel fibrin sealant porcine abrasion studies (Vivostat™ spray only) compared to controls.

Note: ↓ = decreasing volume; ↑ = increasing volume.

* = P<0.05; ** = P<0.01; *** = P<0.001.

■ = significant difference/expected significant difference.

In addition to rabbit and porcine models a recent study has been conducted investigating PSA development and prevention with novel fibrin sealant in a rat caecum to peritoneum abrasion model. Treatment with novel fibrin sealant significantly reduced the formation of PSAs by 80.2% (Graph 10.1; Appendix X Table 10.2).



Graph 10.1: Mean volumes of PSAs, fibrin sealant remaining and fibrin sealant associated reaction
together with SEMs and statistical differences.

It has been shown that Vivostat™ system novel fibrin sealant significantly reduces PSA development in 3 different animal species - rabbit, pig and rat. In addition novel fibrin sealant significantly reduces PSA development at uterine horn, stomach, colon and

caecal abraded sites to abraded peritoneum in a severe PSA testing model. The clinical implications of PSA prevention at these sites within the peritoneum is great. The main pathogeneses associated with PSAs are intestinal obstruction, infertility and abdominal pain. Approximately 0.69% (Stewart *et al.,* 1987) to 1% (Menzies & Ellis, 1990) of all laparotomies result in intestinal obstruction, with adhesions being the primary cause of intestinal obstruction in the western world (Nemir *et al.,* 1952; Perry *et al.,* 1955; Playforth *et al.,* 1970; Stewardson *et al.,* 1978; Bizer *et al.,* 1981; Menzies & Ellis, 1990; Stricker *et al.,* 1994). This condition is associated with mortality ranging from 0 to 37% depending upon adhesion type, patient age, surgical complications, duration before treatment and types of treatment received (refer to Section 1.1.3.7). Whereas 15-20% of female infertility is attributed to adhesions (Soules *et al.,* 1982; Hull *et al.,* 1985) and their presence is also thought to be responsible for some cases of chronic pelvic pain (Kersch *et al.,* 1984). The prevention of PSAs at these sites within the peritoneum would lend to reduced patient mortality and morbidity.

PSA prevention would have great economic and workload benefits. In 1994 $1.3 billion was spent on adhesiolysis in the Unites States (Ray *et al.,* 1998). A previous study by Ray *et al.* (1993) demonstrated that the rate of adhesiolysis, approximately 1% of admissions, had remained stable in the US between 1988 and 1994 even with the increased use of laparoscopic techniques. In the UK there are 8400-9600 admissions each year for adhesional intestinal obstruction with half of these actually undergoing surgery (Scott-Coombes *et al.,* 1993). With an average length of hospitalisation being 15 days (McEntree *et al.,* 1987) this is a large drain on the National Health Service. Any agent which could prove consistent PSA prevention would, within limits, be economically viable.

With regard to PSA prevention in the past, many different PSA prevention agents have been tried ranging from separation of surfaces by foils of metal (Boys, 1942; Ellis, 1982), hydroflotation by hypertonic solutions (Pijlman *et al.,* 1994; Risberg, 1997) to inhibition of fibroblast proliferation via cytotoxic drugs (Ellis, 1971; Ellis, 1982). Most of these agents tried have induced PSA development rather than reduced incidence, due to inflammatory response induction. In addition many of the chemical agents have

associated non acceptable side effects (Examples: dextran (Holtz, 1984; DiZerega, 1994; Pijlman *et al.,* 1994; Risberg, 1997), corticosteroids (DiZerega, 1994; Pijlman *et al.,* 1994; Risberg, 1997) and heparin (Connolly & Smith, 1960; Ellis, 1971; Pijlman *et al.,* 1994)).

Much greater success has been achieved with the less drastic methods of PSA prevention, that is the use of physical barriers to isolate damaged surfaces. The most successful artificial non-absorbable barrier to date is Gore-Tex surgical membrane (W.L. Gore & Associates, Flagstaff, AZ, USA) however, the main limitation with this product is the need for it to be held in place via sutures as a permanent foreign material (DiZerega, 1994; Risberg, 1997). This is probably responsible for the conflicting reports for PSA prevention although most clinical studies have yielded positive results (DiZerega, 1994; Pijlman *et al.,* 1994).

Of the artificial absorbable barriers Interceed® (Johnson & Johnson Medical Inc., Arlington, TX, USA) and Seprafilm™ (Genzyme Corporation, Cambridge, MA, USA) have demonstrated the greatest PSA reduction potential. Seprafilm™ has regulatory approval for prevention of PSA formation in many European countries and North America and received a lot of attention when it was first available. However both of these products have technical limitations: Interceed® requires removal of intraperitoneal irrigants, excess peritoneal fluid and blood and Seprafilm™ needs to be kept completely dry, handled gently with dry hands and tissue contact avoided until correct application site reached. These problems have lead to limited success of these products in the clinical surgical field.

Fibrin sealants provide an alternative, natural, absorbable barrier system. There are currently five fibrin sealants commercially available - Tissel®, Beriplast®, Biocol®, Bolheal and Quixil, with several others in the clinical pipeline (Table 10.5). These products have been developed primarily as haemostats with studies and regulatory approval concentrating on this aspect and, to a lesser extent on tissue sealing. However these may not be the most appropriate applications for fibrin sealant, with further applications in wound healing and being a carrier medium for cells, antibiotics and

chemical mediators presenting potential applications.

Fibrin Sealant	Company	Regulatory Approval
Products on the market		
Tisseel®	Immuno	1978 (Germany) 1998 (US)
Beriplast®	Centeon	1983 (Germany)
Biocol®	LFB - Lille	1984 (France)
Bolheal	Chemo-Sero Inst.	(Japan)
Quixil	Omrix	1997 (Israel)
Products in clinical pipeline		
Fibriseal/Sealgen	American Red Cross/Baxter	Expected
Haemaseel	Haemacure	Clinical trials
SNTB Fibrin Sealant	SNTB	Pending
ViGuard	Vitex	Clinical trials

Table 10.5: Fibrin sealants
(Taken from Brodniewicz, 1998).

There are very few clinical studies which have investigated the potential of fibrin sealants to prevent PSAs (Larsson *et al.,* 1986; Brands *et al.,* 1990; Alnot *et al.,* 1993; Izbicki *et al.,* 1994). Overall, pre-clinical data supports the use of fibrin sealants for PSA reduction (Lindenberg & Lauritsen, 1984; Larsson *et al.,* 1986; Koltai & Gerhard, 1990; Dulchavsky *et al.,* 1991; Sheppard *et al.,* 1993; De Laco *et al.,* 1994) although conflicting reports do exist (Bilgin *et al.,* 1995). The study investigating Tissucol (Tisseel®) prevention of PSAs contained in this thesis demonstrated that it did not significantly reduced PSA formation and that, with 4 units of thrombin, actually increased PSA volume compared to controls. This has previously been seen with prior in house studies. This study did, however, use bovine thrombin which was associated with a reactive response and change to the use of pooled human blood derived thrombin with Tissucol®, although not ideal, will probably be associated with reaction with a reduced reaction magnitude, and potentially, increased PSA reduction properties. The exclusion of animal proteins and anti-fibrinolytics currently used in fibrin sealants is an emerging issue. During the studies of this thesis it has been highlighted numerous times that inflammation has the effect of inducing PSA formation and any agent which causes an additional inflammatory response will hinder PSA prevention. In fact the use of non-

autologous, thrombin fibrin sealants is associated with tissue fibrosis (Fairbrother, 1997) which is antagonistic to PSA prevention.

The overall reduction of PSAs using novel fibrin sealant (automated preparation and application) for studies (rabbit, pig and rat) reported in this thesis ranged from 59-99%, with an overall mean of 79%. These results have been determined using very strict and unbiased quantitative analyses and no other study available for our reference has used such a strict system to assay potential PSA preventative agents. Previous studies have lent support for many potential PSA preventive agents, especially fibrin sealants (Tisseel®, cryoprecipitate), Seprafilm™, Interceed® (TC7), and tissue plasminogen activators (refer to Appendix I Table 1.3). Although many of these studies report a significant reduction in PSA formation their qualitative or semi-quantitative methodology prevents direct comparisons with studies investigating novel fibrin sealant. The consistent reduction to near complete prevention of PSAs using novel fibrin sealant appears to highlight that it is the best PSA preventative agent developed at present.

It is a perverse concept that fibrin, which is responsible for the formation of fibrinous and ultimately fibrous PSAs, is also the substance which has demonstrated the most promise for preventing them. In fact it was initially thought that fibrin sealants would form a fibrin matrix which would allow fibroblast migration, increasing the potential for PSA formation (Sheppard *et al.*, 1993). This is not the case, as fibrin sealants form a barrier which is generally inaccessible to host cells. Since fibrin is naturally present in these situations and artificial fibrin possesses very similar properties to natural fibrin, resulting in haemostasis and forming a temporary protective covering over damaged surfaces, application of fibrin sealant mimics the first stages of wound healing without the meshwork for fibrous PSA formation being established. This supports Lindenberg & Lauritsen (1984) who postulated that fibrin sealant may seal lymph and blood vessels and thus prevent fibrin exudate from injured surfaces.

During the course of these studies, both rabbit and porcine abrasion models have been utilised. The development and prevention of PSAs in the rabbit model have been extensively studied from 1 hour to 42 days post injury. Less analyses have been

conducted in the porcine model however the two studies performed sampled tissue at 7 days (pilot study) and 14 days (main study) following injury. Although the porcine data at 7 days is limited by small sample numbers it does give us some insight into the dynamics occurring throughout this time window. For control cases, PSA volume increased by 3.72 fold from 7 to 14 days whereas inflammation volume decreased by 6.24 fold. Treated cases demonstrated a similar pattern with PSA volume increasing by 1.62 fold and inflammation volume decreasing by 1.48 fold from 7 to 14 days. In addition, volume of fibrin sealant remaining decreased by 6.98 fold. This suggests that PSA maturation is still ongoing from 7 to 14 days with the volume increase most probably due to collagen deposition by fibroblasts. However, the inflammatory response to injury is decreasing throughout this time, with tissue repair predominant.

This follows the overall trend seen in the rabbit model but there is a difference with regard to PSA volume dynamics. In the rabbit model from 7 to 14 days in both control and treated cases mean volume of PSA decreased by 1.17 fold and 1.39 fold (total tissue generation) respectively. Although this could be due to species differences it is more likely due to differences in PSA induction sites. Previous studies have demonstrated that fibrinolytic capacities of different organs vary significantly in humans (Albrechtsen, 1957; Sandberg *et al.,* 1963) with large amount of plasminogen activator present in uterine tissue (Albrechtsen, 1957). Marshall (1985) compared the levels of extractable fibrinolytic activity within human tissues (Appendix X Table 10.3) and demonstrated that tissue intrinsic fibrinolytic abilities differ, with uterus having the greatest capacity, followed by colon and finally stomach. This demonstrates an inverse relationship with the results obtained by the studies within this thesis, with PSAs greatest at stomach, colon, and uterine horn experimental sites respectively. Hence the tissues own fibrinolytic capacity influences the degree of PSA formation. With regard to PSA prevention with novel fibrin sealant, PSA presence was significantly reduced at all sites but followed the same pattern as control sites (stomach, colon and uterus respectively). This could be due to tissue fibrinolysis and/or due to fibrin sealant coverage, either during application or subsequently influenced by organ expansion and motility. This increased fibrinolytic ability could decrease the time required for tissue modelling and hence promote earlier tissue remodelling, producing the results demonstrated in our

studies.

All fibrin sealants currently commercially available are non-autologous with the use of pooled human fibrinogen and exogenous proteins such as thrombin, calcium ions and aprotinin. The use of such agents leads to potential viral contamination and antigenicity problems. In the studies in this thesis the use of non-autologous novel fibrin sealant was associated with an acute inflammatory response even though no exogenous proteins were present. However this inflammatory response was absent in autologous novel fibrin sealant with only local trauma tissue reaction present. Although data for autologous and non-autologous novel fibrin sealant is limited to the results of one pilot study, PSA prevention varied greatly between them with reductions of 99% and 59% respectively. Although differences in efficacy can be attributed to experimental differences such as sealant application, the reduced volume of inflammation in the autologous group (16 fold less) lend support for this being a true difference in PSA prevention potential. We know from this data and others that PSA severity is associated with damage severity (Ellis, 1962; Belzer, 1967). Inflammation will induce damage and is therefore associated with severity of PSA formed. Increased inflammation, whether trauma induced or foreign material induced will lead to a greater severity of PSAs, that is, greater incidence and greater volume. Autologous fibrin sealants which do not induce an inflammatory response are a much more desirable PSA preventive agent.

Angiogenesis plays a pivotal role in wound healing accounting for up to 60% of repair tissue (Dyson *et al.*, 1991). Since all tissues require a circulatory system for the delivery of nutrients, cells and chemical mediators, rapid formation of blood vessels at injury sites occurs both with adhesion-free healing and PSA development. Preliminary data concerning length density and total length of blood vessels within PSAs and tissue generation in both control and novel fibrin sealant treated cases was obtained via stereological analyses (Appendix X Table 10.4).

The first new blood vessels were seen at 1 day post injury in both control and novel fibrin sealant treated cases using a rabbit uterine horn abrasion model. This is very rapid generation of vascular tissue, which has previously been associated with PSA

development (in-house observation). Since these results are very preliminary interpretation is cautious, however it does appear that cases treated with novel fibrin sealant have reduced vessel length density and total length compared to control cases. This is probably related to reduced inflammation and injury tissue generated in novel fibrin sealant treated cases.

The studies within this thesis have been concerned with abdominal adhesions and therefore the discussion has concentrated on this PSA type. However, adhesion formation causes problems with many surgical therapies, especially tendon, pleural and cardiac. Successful repair of divided digital tendon continues to remain a surgical challenge primarily due to adhesion development. Poor surgical technique may actually accentuate previous tendon damage with additional adhesion induction. Adhesion presence prevents the tendon from sliding freely within its sheath and finger function is severely impaired with associated weakness and stiffness (Bucknall & Ellis, 1984). Prevention of this type of adhesion would have great patient benefits and in turn economic relief from re-operations and physiotherapy.

The formation of pleural and cardiac adhesions also have serious consequences. Pleural adhesions can potentially lead to restrictive disturbances of ventilation and partial respiratory insufficiency (Kaiser *et al.*, 1979) whereas the main problem with cardiac adhesions is with reoperation where there is an increased risk of damage to the heart during resternotomy (Milgalter *et al.*, 1985). This ultimately limits the number of operations which can be safely attempted. Since peritoneal, pleural and cardiac cavities have several physiological similarities: delicate surface covered with a single layer of loosely connected mesothelial cells bathed in an extracellular fluid, it is highly probable that these membranes undergo matching processes during PSA development. Therefore PSA prevention should be able to be tackled in a like manner.

Although extensive assays were performed using TNF-α and TGF-β ELISA kits purchased from Genzyme Diagnostics inaccurate and non-reproducible results were obtained using control samples. Since specific anti-rabbit commercial kits are not available, alternatives were used: an anti-mouse TNF-α ELISA kit and anti-human

TGF-β ELISA kit. Since no cross reactivity data comparing these kits with rabbit samples was available, amino acid homologies were compared. The amino acid homology of mouse and rabbit TNF-α is 77.87% (data obtained from Genzyme Diagnostics) and 98% for species homology of TGF-β species (Massague, 1990; Kondaiah *et al.,* 1990). Despite the high amino acid sequence homologies these kits failed to detect rabbit TNF-α and TGF-β. Thus the antigen used for the detection of these cytokines was either absent or altered making it unavailable for the antibody coated on the base of the ELISA kit. This non-detection of rabbit cytokines by these kits was not totally unexpected as these kits are designed to be highly specific for their antigen of choice, with cross-reactivity actively avoided in their design.

For the detection of fibrin in formalin fixed, paraffin wax embedded rabbit tissues an anti-human fibrin IgG1 (American Diagnostica Inc, Greenwich, USA. Supplied by Alpha Laboratories Ltd, Eastleigh, Hampshire, England) was assessed and human (Interstitial) collagen I, II, III clone MCI-HA monoclonal antibody (Monosan, Netherlands, supplied by: Bradshaw Biologicals Ltd, Loughborough, England) was assessed for collagen detection. Although these anti-human antibodies were the most promising at the time of these experiments, both failed to detect rabbit proteins even with protocol modifications in primary antibody incubation time and temperature and alteration of proteinase incubation for fibrin detection. As with the ELISA failures this was due to the antibody directed against an epitope which is either not present on the rabbit proteins or not available.

Analyses of the collagen antibody to detect rat tail collagen (performed by Dr J Featherstone, Oxford BioResearch) found that at a dilution of 1:10 collagen was detected, however negative results were found at a dilution of 1:30. The usual concentration of this antibody used in immunohistochemistry in this study was 1:20. This dilution may have been too weak. However the concentrated nature required for detection brings the quality of this antibody into question.

10.1 Study Limitations

During the course of the work for this thesis several limitations were evident:

i. The use of static time points to interpret a dynamic process.

Although the rabbit PSA development and prevention studies sampled PSAs and surrounding tissues extensively throughout their formation and maturation, a certain amount of data extrapolation is required. Although I feel that these studies provide a precise and unbiased account of the mechanisms occurring changes at time points not sampled must be acknowledged. The presence of these intermediate changes would not change the pattern of PSA development and prevention but may slightly alter the time scale in which these processes are thought to occur.

ii. The use of a rabbit model. This small experimental model has the benefits of being accepted as a model for PSA investigations with utilisation by many studies. However,

a. it prevents the use of novel fibrin sealant as truly intended, a totally autologous fibrin sealant and therefore does not actually use this agent as it will be used clinically.

b. the resultant inflammatory response mediated due to the presence of foreign protein and the undetermined effect of this reaction on PSA development needs to be taken into consideration when analysing prevention of PSAs by novel fibrin sealant and the effect on overall wound healing.

c. the small circulating blood volume in the rabbit complicates the taking of blood for biochemical analyses with marked effects of venesection of blood cell profiles.

d. the inconsistency of rabbits with regard to blood factors (Schermer, 1967) is also a fact to be aware of in analysis of these studies.

e. the lack of uniform blood cell nomenclature requires specific study definitions.

f. the lack of commercially available antibodies directed against rabbit antigens makes very precise biochemical and immunohistochemical analyses very difficult.

iii. The sampling technique for tissues taken to resin made assumptions concerning cellular distribution throughout PSAs and tissue generation with the middle portion sampled only. Although practical reasons governed this procedure this should be avoided in subsequent studies. I do not feel that this invalidates the results obtained since all tissue will treated equally and the conclusions drawn, that is, cellular presence throughout PSA development, PSA prevention with novel fibrin sealant and comparison between the two, are unaffected. However if this work was to be directly compared to other work of this kind (of which there is currently none) then this sampling regime would have be acknowledged.

iv. Since tissue zone volumes were calculated from wax sections and cell number counting was performed using resin sections processing differences may exert an affect on number densities. Comparison of wax and resin sections as concurrent sections for estimation of volumes of uterine horn width and height, peritoneum height, PSA, PTG and UTG, showed a mean percentage difference between wax and resin of 14% for all parameters and 27% for tissue generation only (PSA, PTG and UTG) (Appendix X Table 10.5). Since these volume comparisons were taken from concurrent sections (for example, L3 (wax), L4 (resin) and L5 (wax)) and not the same sections, these percentage differences are within the limits of biological variation. Although a difference may exist in shrinkage between resin and wax processing it only exerts a minor effect on cell number densities for these studies.

v. 25μm resin sections were used for cell number analyses and consistent staining throughout the whole section thickness proved difficult. The main problem was the inadequate staining of eosinophilic granules with some staining very pale pink. This may have potentially lead to incorrect differential classification of eosinophilic and basophilic cells. For this reason eosinophilic and basophilic cells were grouped together as granulocytes for comparisons between PSA development and prevention with novel fibrin sealant.

10.2 Conclusions

i. Studies contained within this thesis have demonstrated that PSA development is a complex, multi-factorial highly regulated process. This is the first study to identify that PSA development has two distinct stages:

a. PSA modelling: Fibrinous PSA formation and maturation (1 to 16 hours)

Including - fibrin deposition and maturation
- inflammatory response
- initiation of clearance of necrotic tissue

b. PSA remodelling: Fibrous PSA formation and maturation (1 to 14 days)

Including - collagen deposition and maturation
- completion of necrotic tissue clearance
- tissue repair.

ii. Prevention of PSA development by fibrin sealants is now a reality with Vivostat™ system human derived (novel) fibrin sealant. Treatment of surgical injuries with this sealant resulted in repair tissue generation discretely located on injured surfaces, not adhering to other surfaces. Tissue generation development proceeded in a similar manner to PSA development with two stages seen:

a. Tissue generation modelling: Fibrinous tissue generation formation and maturation (1 hour to 1 day)

Including - fibrin deposition and maturation
- inflammatory response
- initiation of clearance of necrotic tissue

b. Tissue generation remodelling: Fibrous tissue generation formation and maturation (1 to 7 days)

Including - collagen deposition and maturation
- completion of necrotic tissue clearance
- tissue repair.

iii. Treatment with novel fibrin sealant reduced the amount of injury-associated inflammation, necrotic tissue damage and repair tissue generated. The net result was improved, more rapid tissue repair.

iv. PSA prevention and acceleration of wound healing by novel fibrin sealant appears to occur as a result the sealant acting as a haemostat and as a physical barrier. Thus reducing the body's response to injury and preventing the exudation of fibrin and other tissue/serous fluids from abridging injured surfaces and interacting with surfaces and peritoneal contents.

10.3 Future Research Options

Due to the physical limitations placed upon this thesis with respect to time, a number of research options have not been included which may present avenues along which future studies may be explored.

i. Further understanding of biochemical mechanisms in PSA development and prevention with novel fibrin sealant.
ii. Further work on autologous novel fibrin sealant (porcine novel fibrin sealant).
iii. Prevention of secondary PSAs by novel fibrin sealant.
iv. Prevention of cardiac PSAs by novel fibrin sealant.
v. Prevention of tendon PSAs by novel fibrin sealant.
vi. Prevention of PSAs by novel fibrin sealant via laparoscopy.
vii. Further development of Vivostat™ applicator for precise delivery of novel fibrin sealant to small areas.

11.0 References

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APPENDICES

APPENDIX I

Factor	Stimulating migration of endothelial cells	Stimulating mitosis of endothelial cells	Inhibiting migration or proliferation of endothelial cells
Basic fibroblast growth factor	+	+	-
Granulocyte-macrophage colony stimulating factor	+	+	-
Vascular endothelial growth factor /vascular permeability factor	+	+	-
Interleukin 8	+	+	-
Substance P	+	+	-
Angiotropin	+	-	-
Human angiogenic factor	+	-	-
Platelet derived growth factor	? +	? +	-
Interleukin 6	? +	-	? +
Interleukin 1	? +	? +	+
Transforming growth factor-beta	low +	low +	high +
Tumour necrosis factor-alpha	low +	-	high +
Transforming growth factor-alpha	-	+	-
Insulin-like growth factor	-	+	-
Interferon-alpha	-	-	+
Interferon-gamma	-	-	+
Thrombospondin 1	-	-	+
Monocyte-derived endothelial cell inhibitor	-	-	+
Macrophage-derived endothelial cell inhibitor	-	-	+

Table 1.1: Macrophage Derived Mediators of Angiogenesis
(taken from Sunderkotten *et al.*, 1994)

CELL TYPE	PRIMARY LOCATIONS	KINETICS	FUNCTIONS
Neutrophils	• Blood - circulating/loosely adhered to vascular endothelium • Tissues	• Spend ~10 hours in blood before passing into tissues • No evidence that tissue neutrophils re-enter blood • Migration into extra-vascular or tissue space accelerated by damage to the vessel or inflammation	• Bacterial killing (chemotaxis, opsonization, ingestion & killing) • Involved in inflammation • Synthese/release of: histamine, heparin's, neutral protease's, IL-1,3,4,6, INF-gamma, CM-CSF, neutrophil chemotactic factor, PAF, ?TNF-alpha, angiogenesis factors
Eosinophils	• Blood • Tissues	• Half life of 4-5 hours in circulation • Tissue life span of 8-12 days • Some re-circulation between tissues and blood	• Bacterial killing (not as potent as neutrophils) • Inactivation of mediators released by tissue mast cells degranulation (reduction of body's response to IgE mediated reactions) • Limiting some parasitic infections • Release TGF-alpha/beta in response to wound healing • ? Important role in organisation of wound healing
Basophils	• Blood	• Motile; attracted by chemotaxins such as complement components and products of activated lymphocytes (lymphokines)	• Anaphylaxis • Cell mediated hypersensitivity • Local inflammatory response • weak phagocytic activity • considerable pinocytic capability • histamine & heparin content, IgE binding & degranulation
Lymphocytes	• Blood • Lymphatics • Secondary lymph organs - nodes, spleen, alimentary canal	• Blood: 70-80% T cells & 15-20% B cells • Short and long lived population • Mean survival time from 4-10 years	• B Cells • Formation of specific immunoglobulins • T Cells • Cell mediated immune responses • Induce angiogenesis via ECM proteins and is the effect of different angiogenic stimuli
Monocytes	• Blood - circulating & marginal pools • Tissues (macrophages)	• An intermediate stage in the development of the tissue macrophage • Sluggishly motile • Spends 10-12 hours in circulation before migrating into tissues • Increased enzyme activity and number of mitochondria • Capable of further proliferation • Life span of several months	• Microbial killing (chemotaxis, phagocytosis, oxygen-dependent & hydrogen peroxide systems) • Removal of debris • Immune response (processing antigens for easier identification) • Delayed hypersensitivity reactions • Involved in inflammation • Capable of secreting PDGF, TGF-α& β, IL-1-α& β, IL-6,8, GM-CSF, G-CSF, M-CSF, TNF-α, IFN-γ, ?EGF • Potent at enhancing peritoneal trauma cell proliferation • Involved in angiogenesis (interplay of positive & negative mediators)
Platelets	• Circulation (70%) • Spleen (30%)	• Life span of 8-11 days • Spleen & circulating platelets exchange freely	• Haemostasis (pro/anti-coagulant activity; fibrinolytic activity) • Platelet plug secretes granules (serotonin, adrenalin, ADP, clotting factors) => fibrin clot laid down • Stimulate neutrophil accumulation & degranulation => inflammation • Induce endothelial cell differentiation => capillary-like structures • Involved in tissue repair
Mast Cells	• Loose connective tissue	seen either singly or grouped along with small blood vessels	• Increased numbers in wounds or chronic fibrotic disease • Active during normal wound healing • Pharmacological activation pre-operatively may improve wound healing • Involved in angiogenesis • Play an important role in peritoneal adhesion formation
Fibroblasts	• Connective tissue		• Essential for wound healing and adhesion formation • Syntheses collagen • Plays pivotal role in angiogenesis • Progenitor cell for endothelial cells • Least specialised of the connective tissue cells, with further differentiation possible • Capable of secreting FGF, IL-1α and β, GM-CSF, G-CSF, TNF-α and IFN-β

Table 1.2: Cell types and functions.

Substance	Animal Species	Author(s)	
Prevention of Fibrin Coagulation			
Heparin	Female NZW rabbits	Fukasawa *et al.*, 1991	local IP administration of low dose heparin through the immediate post operative interval may result in adhesion free healing
Heparin	Wistar rats	Chandy & Rhoads, 1946	did not influence adhesion formation
Heparin hydrated collagen shield	Rabbit	Murray *et al.*, 1990	may prevent post operative fibrin formation in eyes
Heparin and/or sodium CMC	Female Wistar Albino rats	Sahin & Salaam, 1994	SCMC alone produced significant reduction in PSAs. However more favorable adhesion was achieved with SCMC and low molecular weight heparin compared to SCMC alone, without any haemorrhagic complications
Histadyl®	White rats	Jacqmain & Shumacker, 1962	diminishes experimental produced peritoneal adhesions
Reduction of Inflammatory Response			
Anti-Inflammatory Drugs			
Promethazine (IM) and/or Methylprednisolone (IM)	Macaca monkeys	Liao *et al.*, 1973	steroids are effective in a fresh wound in preventing adhesions. However prevent of recurrent adhesion seems only effective if adhesions have undergone maturation. Steroid treatment during early maturation of the wound appears to have little, if any, effect in preventing adhesion formation
Tolmetin	Female NZW rabbits	Rodgers *et al.*, 1990	continuous delivery of small amount directly to the injury site lead to significant reduction in the number and severity of adhesions. A single IP injection required relatively high concentration to prevent adhesion formation. Acid form of tolmetin was more potent than sodium salt to prevent adhesion formation
Pentoxifylline	Female NZW rabbits	Steinleitner *et al.*, 1990	marked inhibition of adhesion reformation after lysis of pelvic adhesions
Methylprednisolone	Dogs	Vander Salm *et al.*, 1986	the near total elimination of pericardical/epicardial adhesions utilizing methylprednisolone, is also achieved in humans, would markedly reduce the difficulty and increase safety of cardiac operations
Hydrocortisone	Human (women)	Jansen, 1985	no empiric basis to support use to prevent peritoneal adhesions
Combination with histamine antagonists			
Combined Promethazine + Dexamethasone	Rats and dogs	Replogle *et al.*, 1966	use of an antihistamine, promethazine (Phenergon®) and an adrenocorticol hormone dexamthasone (Decadron®), the inflammatory phase that follows trauma to intestinal serosa can be minimized and the organization of inflammatory exudate into fibrous adhesions can be delayed
Combined Promethazine + Dexamethasone	Male SD rats	Grosfeld *et al.*, 1973	although promethazine and dexamethasone treatment clearly reduces the incidence of intestinal adhesions, signficant morbidity and mortality rates question their routine application. Such therapy should be avodied in instances of potential or overt peritoneal sepsis.
Calcium Channel Blockers			
Verapamil	Female NZW rabbits	Steinleitner *et al.*, 1989	marked inhibition of adhesion reformation after lysis of pelvis adhesions
Verapamil	Female NZW rabbits	Steinleitner *et al.*, 1990	potently modulates peritoneal healing and regeneration, and significantly reduces adhesion formation. IP delivery and systemic administration appear equipotent in this model
Vitamin E	Feamle Swiss White mice	Kagoma *et al.*, 1985	significant decrease in incidence and degree of adhesions with vitamin E supplements
5-Fluorouracil (anti-metabolite)	Male Albino Charles River rats	Goldman & Rosemand, 1967	formation of adhesion was prevented using 50mg/kg subcutaneously per day, with marked toxicity associated with this dose
Removal/Dissolution of Deposited Fibrin			
Fibrinolytic Agents			
Recombinant tPA	SD rats	Evans *et al.*, 1993	levels required to alter or prevent intra-abdominal adhesion formation also produce significant impairment of early phase of wound healing as measured by wound content of hydroxyproline
Recombinant tPA	Female NZW rabbits	Dunn & Mohler, 1990	direct application of tPA gel inhibits PSA formation in a rabbit model, probably related to a local increase in fibrinolytic activity
Recombinant tPA	Female NZW rabbits	Menzies & Ellis, 1989	rtPA ia an effective inhibitor of adhesion formation in the experimental model
Recombinant tPA and CMC	Female NZW rabbits	Gehlbach *et al.*, 1994	Combination of rtPA and CMC did not reduce PSA reformation and was associated with haemorrhagic complications
Ancrod	Mongrel dogs	Buckman *et al.*, 1975	IV administration prevents visceral post operative adhesions in the dog, with infrequent serious haemorrhagic complications when correctly administered
Thrombin (Thrombostat)	Female SD rats	McGaw *et al.*, 1988	thrombin alone does not decrease PSA formation
Fibrinolytic drugs (tPA; tPA analog Fb-Fb-CF), streptokinase	NZW rabbits	Wiseman *et al.*, 1992	tPA analog and streptokinase reduced adhesions and was unaffected by ORC delivery (low extent of post operative bruising and bleeding). TPA and ORC delivery reduced adhesions but was associated with significant post operative brusing and bleeding. Safety of these fibrinolytic drugs is required before they are used clinically.

Substance	Animal Species	Author(s)	
Proteolytic Enzymes			
Papain	SD rats + dogs	Stevens, 1968	no inhibitory effect upon formation of peritoneal adhesions in two species could be demonstrated
Papase (anti-inflammatory)	Monkeys	Kapur *et al.*, 1969	oral administration was found to be very effective in preventing peritoneal adhesions
Papase (anti-inflammatory)	Male Albino rats	Nair *et al.*, 1974	oral administration immediately post operative was found to reduce markedly the incidence and extent of adhesions. Equally effective in reducing the incidence and extent of reformation of adhesion after their lysis
Hylauronidase	Wistar rats	Chandy & Rhoads, 1946	60% adhesion prevention
Separation of Surfaces			
32% Dextran 70	Human (women)	Rein & Hill, 1989	significantly inhibited lymphocyte proliferation and macrophage phagocytosis (but not cell viability). This immunosuppressive effect in vitro suggesting a new mechanism by which 32% dextran 70 may reduce PSA formation
	Human (women)	Adhesion Study Group, 1983	IP high molecular weight dextran was found to reduce PSA formation effectively
	Female NZW rabbits	Soules *et al.*, 1982	degree of adhesion formation did not differ to controls (inconclusive results)
	Male Patient	Krinsky *et al.*, 1984	50ml of Hyskon instilled at time of laparoscopy. 26 hours later at subsequent laparotomy, 255ml of peritoneal fluid was presented, demonstrating that Hyskon was able to promote transfer of a large volume of fluid into the peritoneal cavity. By separating fibrin-coated surfaces, this hydroflotation effect inhibits adhesion formation
	Human (women)	Jansen, 1985	no empiric basis to support the use to prevent peritoneal adhesions
Gore-Tex Surgical Membrane	Female NZW rabbits	Boyers & Jansen, 1990	mean scores for extent, type and tenacity of adhesions were significantly lower for Gore-Tex covered lesions. Gore-Tex covered defects also reformed adhesions at a significantly lower rate than controls.
	Female Mongrel dogs	McCabe Fowler *et al.*, 1991	appears to increase post-radical surgery adhesions
Silastic (silicone rubber)	Mongrel dogs	Youmons *et al.*, 1968	silastic prevented adhesions, both pleural and pericardial, and was not implicated in a single case of infection
Carboxymethylcellulose (CMC)	Female NZW rabbits	Heidrick *et al.*, 1994	SCMC if effective in preventing PSAs formation in NZW rabbits
	Male SD rats	Ryan & Sax, 1995	more effective than Interceed® in preventing PSAs. Action not due to inhibition of TGF-β expression or macrophage derived fibrinogenic factors. Highlights the importance of local barrier effect in adhesion prevention.
Sodium CMC + Vitamin E	SD rats	Hemadeh *et al.*, 1993	synergistic beneficial effect of oral supplements of Vitamin E and IP administration of SCMC solution in reducing the incidence and degree of IP adhesions
Oxidised Regenerated Cellulose (ORC)			
Resoluble hydrogel barrier	Female NZW rabbits	Hill-West *et al.*, 1994	highly effective for reduction of periovarian adhesions in this model
Oxycel	Male SD rats	McGaw *et al.*, 1988	some benefits in decreasing PSA formation in the rat
ORC	Female NZW rabbits Male Mongrel dogs	Shimanuki *et al.*, 1987	graded reduction in adhesion formation and significantly prevented adhesion reformation
Interceed® (TC7)	Female rabbits	Diamond *et al.*, 1990	Interceed® (TC7) significantly reduces postoperative adhesion formation. The material is resorbed by the body, presumably by intraperitoneal processes over a very short time, and without evidence of typical foreign body reaction
Interceed® (TC7)	Human (Female)	Malinak, 1990	Interceed® adhesion barrier effectively reduced the incidence, extent and severity of postoperative adhesion.
Barrier methods with/without Dextran 70	Female NZW rabbits	Soules *et al.*, 1982	barriers used: Gelfilm, Surgicel, Silastic, Gelfoam paste, amnion, peritoneum and omentum. Generally increased formation of adhesion was seen with these barrier methods compared to control or dextran treated animals
hydrophilic polymer solutions (polyvinylpyrroliodine & a methylcellulose derivative)	Mongrel dogs	Duncan *et al.*, 1988	polyvinylpyrroliodine & a methylcellulose derivative as intraoperative irrigating solutions significantly reduce pericardial adhesion formation compared to Ringer's Lactate
Pericardial meshing	Mongrel dogs	Milgalter *et al.*, 1985	pericardial meshing is superior to the pericardial substitutes examined (Silicone; Bovine) as adhesions and epicardial reactions are significantly reduced and the coronary anatomy is readily identifiable

Substance	Animal Species	Author(s)	
Seprafilm™			
	Rats	Alponat *et al.*, 1997	Seprafilm™ has the potential to lessen adhesion-related morbidity following incisional hernia repair
	Rat, rabbit, dog, chicken	Burns *et al.*, 1997	Seprafilm™ is safe and effective in reducing postsurgical adhesions
	Humans	Beck, 1997	Seprafilm™ is safe and significantly reduces the incidence, extent and severity of postoperative adhesions to the midline incision
Hyluronic Acid (HA)	Mongrel dogs	Mitchell *et al.*, 1994	HA solutions are efficacious in the prevention of pericardial adhesions in this model, and appear safe in doses five times the amount needed to prevent adhesions
	Human	Longaker *et al.*, 1991	midgestation fetal wound healing is characterized by healing without fibrosis or scar formation, probably due to prolonged presence of hyaluronic acid in the matrix of fetal wounds (compared to adult wounds). Prolonged application of hyaluronic acid or hyaluronate protein complexes to wounds in children or adults may modulate healing in a manner that would make the wounds more fetal-like
Fibrin Sealants			
Tisseel® (Immuno)	Male SD rats	Bothin & Hallberg, 1992	decrease adhesion formation to some extent but only when applied in large amounts
Autologous fibrin gel + thrombin and calcium chloride	Male SD rats	Dulchavsky *et al.*, 1991	significant improvement in adhesion formation and intra-abdominal abscess rates
Modified fibrin gel (Immuno) + angiogenic growth factors	Lewis rats	Fasol *et al.*, 1994	feasibility of initiating site-directed formation of new blood vessel structures to the heart
Fibrinogen coat	Rats	Koltai & Gerhard, 1990	prevented extended adhesions
Non-autologous human fibrinogen (Immuno)	Human	Kram *et al.*, 1988	effective at establishing haemostasis and tissue sealing; helpful adjunct in the care of surgical patients
Tisseel® (Immuno)	Male SD rats	Kucukaydin *et al.*, 1995	potentially useful adjunct to vasovasostomy
Tisseel® (Immuno)	Women, rabbits, pigs, monkeys	Larsson *et al.*, 1986	strengthened tubal anastomoses and prevented PSAs
Tissel® (Immuno)	Male Wistar rats	Lindenberg & Lauritsen, 1984	prevents intra-peritoneal adhesion formation in the present rat model
Tissel (Immuno)	Wistar rats	Lindenberg *et al.*, 1985	less adhesions to defects covered with thick layer of fibrin sealant having high or low antiplasmin compared to defects with only thin layer of sealant or control rats
Human fibrinogen (Cryo ppt) + bovine thrombin and calcium	SD rats	Sheppard *et al.*, 1993	serves as a biological dressing that protects against adhesion formation in the abdomen during the initial healing process and that through this inhibition, there is a marked decrease in the amount of adhesions, even at long-term follow-up
Human fibrinogen + bovine thrombin and calcium	SD rats	de Virgilio *et al.*, 1990	(i) inhibits intra-abdominal adhesion formation in rats; (ii) inhibitory action is dependent upon the fibrinogen concentration; (iii) adhesion are reduced regardless of whether the peritoneal defect is closed
Autologous fibrinogen (ethanol ppt) + thrombin, calcium chloride and aprotonin	Female Danish White rabbits	Weiss-Fogh *et al.*, 1990	adhesion formation absent in reanastomosed tubes with autologous fibrin adhesive
Autologous fibrinogen (ethanol ppt) + thrombin, calcium chloride and aprotonin	Female Danish White rabbits	Weiss-Fogh *et al.*, 1993	autologous fibrin adhesive was absorbed after a week and uncomplicated healing observed. Heterogenous fibrin adhesive caused a granulomatous inflammation interpreted as an immune reaction to the host of the foreign protein and conventional suturing resulted in severe tissue damage with an intensive inflammatory reaction
Tissucol® (Immuno)	Female Hibridum Provisal White rabbits	De Laco *et al.*, 1994	preventative effect on de novo PSAs during laparoscopy
Pooled human fibrinogen (Immuno)	Female NZW rabbits	Bilgin *et al.*, 1995	ovarian reconstruction with fibrin glue does not reduce PSA when compared with usual suturing technique

Table 1.3: Potential PSA preventative agents.

Key:

---- Single product investigated with "positive" results

---- Combination therapy investigated

---- "Negative" results

APPENDIX II

2.10 Histology - Paraffin Wax

2.10.1 Processing

Tissue-Tek® VIP 2000 Tissue Processor (Bayer Diagnostic, Basingstoke, UK)

Routine Schedule

Reagent	Incubation Time
10% formal saline	2 Hours
70% alcohol	1 Hour
Absolute alcohol	1 Hour
Absolute alcohol	1 Hour
Absolute alcohol	1 Hour
Absolute alcohol	1 Hour
Absolute alcohol	1 Hour
Xylene	30 Minutes
Xylene	1 Hour
Xylene	30 Minutes
Wax 1	30 Minutes
Wax 2	1 Hour
Wax 3	1 Hour
Wax 4	1 Hour

2.10.4 Haematoxylin and Eosin Staining

Shandon Varistain 24-4 (Shandon, Southern Products Ltd, Astmoor, Runcorn, Cheshire, England)

Reagents

- Cole's Haematoxylin

Haematoxylin	0.6g
Saturated aqueous potassium alum	280ml
1% iodine in 95% alcohol	20ml
Distilled water	100ml

Haematoxylin was dissolved in warmed distilled water and then mixed with the iodine solution. Alum solution was added and the mixture was brought to the boil and then quickly cooled and filtered. The solution was immediately ready for use but required occasional filtering after storage.

- Eosin Stock Solution

  Eosin 5mg

  Tap Water 100ml

  Working Solution

  Dilute stock solution 1:10 with tap water

- 1% acid alcohol

  1% hydrochloric acid in 70% alcohol

Procedure

Reagent	Time
Xylene	5 minutes
Xylene	2 minutes
Absolute alcohol	2 minutes
Absolute alcohol	2 minutes
70% alcohol	1 minute
50% alcohol	1 minute
Distilled water	2 minutes
Cole's Haematoxylin	10 minutes
Running tap water	2 minutes
1% acid alcohol	3 seconds
Running tap water	4 minutes
0.5% eosin	5 minutes
Running tap water	20 seconds
Absolute alcohol	1 minute
Absolute alcohol	3 minutes
Xylene	2 minutes
Xylene	1 minute
Xylene	1 minute

Results

Nuclei	Blue-black
Cytoplasm	Varying shades of pink
Muscle fibres	Deep pink-red
Red blood cells	Orange/red
Collagen	Pale pink-red
Fibrin	Deep pink

2.15.1 Hema-Tek 200 using modified Wright's Stain for Blood Films

Hema-Tek 200 uses a Hema-Tek Stain Pak, which consists of three reagents: Stain solution (methanol dissolved stain), buffer solution and rinse solution. Slides are placed into the staining machine and pass on a conveyer system, with reagents delivered on the conveyer belt.

Procedure

1. Slides were first immersed in staining solution for approximately 2 minutes for fixation.
2. Buffer solution was then mixed with the stain solution and slides were stained in the resulting solution for approximately 7 minutes.
3. Slides were then rinsed with rinse agent for approximately 1 minute.
4. Slides were then air dried.

2.16.1.1 Validation

TNF-α release from Activated Blood Monocytes

(References consulted: Boyum, 1983; Boyum, 1984; Mattsson *et al*, 1994)

Materials

- Nycodenz-NaCl Separation Solutions

Animal	Conc. NaCl Solution (%)	Volume of NaCl (ml)	Volume of 27.6% Nycodenz Solution (ml)	Density (g/ml)	Osmolarity (mosmol/l)
Rabbit	1.02	2.53	2	1.068	330
Rat	1.08	2.55	2	1.068	340

- Medium 199, enriched with Earle's salt solution (Sigma-Aldrich, Dorset, England)
- Lipopolysaccharide (LPS) solution (250ng/ml)

  LPS Stock Solution (1.25mg/ml) diluted 1:5000 with phosphate buffered saline (pH 7.3).

Isolation of Monocytes from Whole Blood

1. 4-5ml of rat (terminal bleed) and rabbit (non-terminal bleed) blood was collected into EDTA. Home office guidelines dictate that 6% of the animals total blood volume (50-60ml of blood/Kg) can be collected from a non-terminal bleed. On the conservative side this is approximately 3ml blood/Kg body weight.
2. Monocyte count was checked using Coulter counter.
3. Nycodenz-NaCl separation solution was prepared.
4. 3ml EDTA blood was layered over 3ml Nycodenz-NaCl solution.
5. The separation mixture was centrifuged at 600xg for 15 minutes
6. Plasma was cleared down to 3-4mm above the interface between plasma and separation solution.
7. The remaining plasma was collected together with slightly more than half the volume of separation solution.
8. Monocyte count was again checked using Coulter counter.
9. The sample was centrifuged at 800xg for 15 minutes and remove ~1.5ml of supernatant to reduce volume of separation solution and plasma.
10. 1.5ml of pre-warmed (37^0C) medium 199, enriched with Earle's salt solution, was added to the monocyte concentrate, and mixed for 10 minutes to distribute cells through the media.

Activation of Monocyte to Release TNF-α

1. Cells (150µl cell media) were transferred to 96-well flat-bottomed tissue culture plate and incubated in 5% CO_2 for 1 hour to allow adherence.
2. LPS solution was prepared.
3. Non-adhered cells were carefully removed by aspiration (to leave monocyte monolayer).
4. Monocytes were incubated with 100ul of LPS solution in 5% CO_2 at 37^0C for 18 hours.
5. Supernatant was collected and centrifuge at 3000xg for 30 minutes to remove cells.
6. Excess supernatant was subsequently stored ("TNF high control) at -70^0C.

2.16.1.1 Production of Tumour Necrosis Factor Alpha from Whole Blood

(Reference consulted: Desch *et al*, 1989)

Materials

- Whole blood (collected into Heparin 10U/ml final)

  Conforming to Home Office guidelines described in 2.16.1.1

- Lipopolysaccharide [Escherichia Coli serotype 055:B5] 10ng/ml

  LPS solution diluted in RPMI-1640 cell culture media (Sigma-Aldrich, Dorset, England)

Method

For each blood sample multiple tubes/plate wells were run

1. Rat (terminal bleed) and rabbit (non-terminal bleed) blood samples were collected into heparin.
2. 225µl of heparinised blood was pipetted into sterile eppendorf tube/cell culture plate well.
3. LPS (10ng/ml) was subsequently added to the blood sample and mildly stirred.
4. The sample was then incubate at 37°C for 4-6 hours.
5. The contents were then collected and centrifuge at 500xg for 6 minutes.
6. The supernatant was then collected and stored at either 4°C for same day analysis or -80°C for future analysis.

2.16.2.1 Validation

TGF-β Extraction from Whole Blood

Preparation of Platelet Concentrate

1. Fresh human and rabbit peripheral whole blood were collected into EDTA (9ml sample). Conforming to Home Office guidelines described in 2.16.1.1.
2. A sample of whole blood was taken for platelet count using Coulter Counter.
3. Whole blood was centrifuged at 220xg for 10 minutes.
4. Platelet rich plasma was then collected and a sample for platelet count using Coulter Counter.
5. Platelet rich plasma was then centrifuged at 1000xg x 15 minutes.
6. Platelet poor plasma was removed, with approximately 0.5ml volume remaining and platelet plug.

7. Platelet concentrate was then placed on agitator and left for at least 1 hour
8. A sample of platelet concentrate was taken for platelet count using Coulter Counter.

Platelet Lysis via Freeze/Thaw Cycle

1. Platelet concentrate was placed into -80°C freezer until thoroughly frozen (approximately 30 minutes).
2. The sample was then thawed at room temperature and TGF-β concentrate collected.

APPENDIX III

Injury Time	Percentage Incidence of PSAs
10 Minutes	87.5
30 Minutes	100.0
1 Hour	75.0
4 Hours	75.0
8 Hours	100.0
16 Hours	100.0
1 Day	100.0
3 Days	87.5
5 Days	100.0
7 Days	75.0
14 Days	87.5
28 Days	100.0
42 Days	87.5

Table 3.1: Macroscopic incidence of PSAs at each time point following injury.

Injury Time		Adhesion	Tissue Generation				Damage Zone		Peritoneal		Uterine Horn Haemorrhage
			Peritoneal	Uterine Horn	Free Floating	Total	Peritoneal	Uterine Horn	Reaction Zone	Cell Conc	
30 Secs	**Mean**	**0.00**	**3.63**	**0.00**	**0.00**	**3.63**	**13.36**	**4.74**	**5.16**	**0.00**	**5.10**
	STD	0.00	3.26	0.00	0.00	3.26	5.37	3.81	3.32	0.00	3.90
	SEM	0.00	1.15	0.00	0.00	1.15	1.90	1.35	1.17	0.00	1.38
	CV%	N/A	31.80	N/A	N/A	31.80	14.20	28.40	22.74	N/A	27.06
1 Min	**Mean**	**0.00**	**2.83**	**0.00**	**0.00**	**2.83**	**4.99**	**4.38**	**10.44**	**0.00**	**4.13**
	STD	0.00	1.38	0.00	0.00	1.38	2.62	1.43	6.24	0.00	2.86
	SEM	0.00	0.49	0.00	0.00	0.49	0.93	0.51	2.21	0.00	1.01
	CV%	N/A	17.25	N/A	N/A	17.25	18.58	11.56	21.14	N/A	24.50
3 Mins	**Mean**	**0.00**	**11.11**	**0.00**	**0.00**	**11.11**	**8.04**	**4.36**	**20.59**	**0.00**	**6.61**
	STD	0.00	8.47	0.00	0.00	8.47	5.09	6.33	6.13	0.00	6.23
	SEM	0.00	3.00	0.00	0.00	3.00	1.80	2.24	2.17	0.00	2.20
	CV%	N/A	26.96	N/A	N/A	26.96	22.39	51.31	10.52	N/A	33.34
5 Mins	**Mean**	**0.00**	**7.10**	**0.00**	**0.00**	**7.10**	**8.17**	**5.31**	**39.25**	**0.00**	**15.18**
	STD	0.00	4.56	0.00	0.00	4.56	3.55	1.93	25.63	0.00	11.64
	SEM	0.00	1.61	0.00	0.00	1.61	1.26	0.68	9.06	0.00	4.11
	CV%	N/A	22.68	N/A	N/A	22.68	15.37	12.82	23.08	N/A	27.10
10 Mins	**Mean**	**0.00**	**19.04**	**0.00**	**0.00**	**19.04**	**10.82**	**3.80**	**77.86**	**0.00**	**11.18**
	STD	0.00	8.87	0.00	0.00	8.87	5.88	5.18	22.45	0.00	7.54
	SEM	0.00	3.14	0.00	0.00	3.14	2.08	1.83	7.94	0.00	2.67
	CV%	N/A	16.47	N/A	N/A	16.47	19.23	48.11	10.20	N/A	23.86
30 Mins	**Mean**	**0.00**	**9.25**	**0.00**	**0.00**	**9.25**	**13.47**	**5.20**	**62.07**	**0.00**	**12.48**
	STD	0.00	1.82	0.00	0.00	1.82	6.23	3.22	16.68	0.00	10.22
	SEM	0.00	0.64	0.00	0.00	0.64	2.20	1.14	5.90	0.00	3.61
	CV%	N/A	6.96	N/A	N/A	6.96	16.35	21.90	9.50	N/A	28.96
1 Hour	**Mean**	**1.74**	**17.04**	**0.69**	**0.35**	**19.82**	**14.31**	**5.96**	**73.95**	**0.00**	**10.72**
	STD	3.54	16.30	1.70	0.77	17.33	8.89	6.82	30.04	0.00	4.32
	SEM	1.25	5.76	0.60	0.27	6.13	3.14	2.41	10.62	0.00	1.53
	CV%	72.06	33.82	87.28	77.16	30.91	21.96	40.44	14.36	N/A	14.24
4 Hours	**Mean**	**3.81**	**18.57**	**6.61**	**0.17**	**29.16**	**8.98**	**8.34**	**95.22**	**0.39**	**10.24**
	STD	6.66	6.63	7.22	0.31	9.87	3.25	7.49	24.84	0.62	4.82
	SEM	2.35	2.35	2.55	0.11	3.49	1.15	2.65	8.78	0.22	1.70
	CV%	61.79	12.63	38.64	65.57	11.96	12.79	31.77	9.22	57.27	16.65
8 Hours	**Mean**	**10.85**	**23.63**	**6.26**	**0.07**	**40.81**	**11.80**	**5.49**	**112.99**	**0.50**	**17.85**
	STD	3.47	6.58	5.02	0.21	7.99	8.28	4.55	30.64	0.97	21.76
	SEM	1.23	2.32	1.78	0.07	2.83	2.93	1.61	10.83	0.34	7.69
	CV%	11.32	9.84	28.36	100.00	6.93	24.81	29.28	9.59	69.28	43.09
16 Hours	**Mean**	**29.53**	**26.59**	**6.92**	**0.09**	**63.12**	**14.77**	**14.11**	**209.64**	**0.16**	**3.13**
	STD	8.38	6.38	4.69	0.25	12.34	7.34	10.60	36.75	0.29	2.20
	SEM	2.96	2.26	1.66	0.09	4.36	2.59	3.75	12.99	0.10	0.78
	CV%	10.04	8.48	23.95	100.00	6.91	17.56	26.55	6.20	65.47	24.82
1 Day	**Mean**	**28.40**	**28.69**	**5.62**	**0.00**	**62.71**	**14.58**	**7.66**	**124.21**	**4.15**	**16.99**
	STD	11.74	11.25	4.68	0.00	16.82	12.11	6.89	23.30	3.35	20.51
	SEM	4.15	3.98	1.65	0.00	5.95	4.28	2.43	8.24	1.18	7.25
	CV%	14.61	13.87	29.42	N/A	9.48	29.35	31.77	6.63	28.57	42.69
3 Days	**Mean**	**42.09**	**63.02**	**6.73**	**2.40**	**114.25**	**19.87**	**5.32**	**218.73**	**34.54**	**5.58**
	STD	42.33	21.01	8.60	6.79	51.14	7.54	5.59	59.19	7.45	12.58
	SEM	14.96	7.43	3.04	2.40	18.08	2.67	1.98	20.93	2.64	4.45
	CV%	35.55	11.79	45.17	100.00	15.83	13.42	37.10	9.57	7.63	79.79
5 Days	**Mean**	**53.06**	**125.86**	**48.54**	**0.57**	**228.03**	**49.65**	**19.13**	**257.58**	**9.14**	**0.08**
	STD	56.88	75.68	22.87	0.72	92.08	30.45	8.60	110.34	11.62	0.24
	SEM	20.11	26.76	8.08	0.26	32.55	10.77	3.04	39.01	4.11	0.08
	CV%	37.90	21.26	16.66	45.00	14.28	21.68	15.91	15.15	44.94	100.00
7 Days	**Mean**	**57.43**	**142.17**	**36.31**	**0.00**	**235.91**	**52.32**	**3.01**	**201.79**	**2.29**	**0.00**
	STD	49.28	87.86	17.02	0.00	116.46	37.47	2.79	106.66	5.69	0.00
	SEM	17.42	31.06	6.02	0.00	41.17	13.25	0.99	37.71	2.01	0.00
	CV%	30.34	21.85	16.57	N/A	17.45	25.32	32.77	18.69	87.74	N/A
14 Days	**Mean**	**48.88**	**239.51**	**10.69**	**0.08**	**299.17**	**67.29**	**2.55**	**213.02**	**0.00**	**0.00**
	STD	49.80	188.94	9.48	0.24	200.02	63.83	4.60	73.71	0.00	0.00
	SEM	17.61	66.80	3.35	0.08	70.72	22.57	1.63	26.06	0.00	0.00
	CV%	36.02	27.89	31.34	100.00	23.64	33.53	63.62	12.23	#DIV/0!	N/A
28 Days	**Mean**	**25.80**	**108.63**	**19.28**	**0.00**	**153.71**	**37.02**	**2.01**	**137.12**	**0.39**	**0.00**
	STD	19.21	178.88	27.94	0.00	182.71	53.90	1.95	31.34	1.10	0.00
	SEM	6.79	63.24	9.88	0.00	64.60	19.06	0.69	11.08	0.39	0.00
	CV%	26.32	58.22	51.23	N/A	42.02	51.47	34.27	8.08	100.00	N/A
42 Days	**Mean**	**38.03**	**69.59**	**27.49**	**0.00**	**135.10**	**14.14**	**1.72**	**146.45**	**0.00**	**0.00**
	STD	34.13	48.07	7.80	0.00	62.11	14.89	1.84	24.35	0.00	0.00
	SEM	12.07	17.00	2.76	0.00	21.96	5.26	0.65	8.61	0.00	0.00
	CV%	31.73	24.43	10.03	N/A	16.25	37.23	37.84	5.88	N/A	N/A

Table 3.2: Volumetric results of tissue zones: mean volumes, SDs, SEMs and CV%.

Injury Time	P VALUE								
	PSA	Tissue Generation			Damage Zone		Peritoneal Reaction Zone	Peritoneal Cell Conc	Uterine Horn Haemorrhage
		Peritoneal	Uterine Horn	Total	Peritoneal	Uterine Horn			
30 Secs - 1 Min	1.0000	0.5340	1.0000	0.5340	0.0014	0.8780**	0.0530	1.0000	0.5813
1 Min - 3 Mins	1.0000	0.0140	1.0000	0.0163	0.1547	0.0830*	0.0055	1.0000	0.5740*
3 Mins - 5 Mins	1.0000	0.2388	1.0000	0.2586	0.9527	0.0499*	0.1300**	1.0000	0.0875
5 Mins - 10 Mins	1.0000	0.0104**	1.0000	0.0104**	0.2937	0.0499*	0.0064	1.0000	0.4276
10 Mins - 30 Mins	1.0000	0.0207**	1.0000	0.0281**	0.3960	0.1610*	0.1328	1.0000	0.7980*
30 Mins - 1 Hour	0.4420*	0.3280*	0.4420*	0.1610*	0.8294	0.7798	0.3447	1.0000	0.6612
1 Hour - 4 Hours	0.4420*	0.8086	0.0104*	0.2064	0.0830*	0.5740*	0.1450	0.2340*	0.8445
4 Hours - 8 Hours	0.0449*	0.1480	0.8780*	0.0212	0.0386	0.3735	0.2233	0.8780*	0.7980*
8 Hours - 16 Hours	0.0002**	0.3756	0.5740*	0.0007	0.4460	0.0529	<0.0001	0.7980*	0.0499*
16 Hours -1 Day	0.8291	0.6541	0.5893	0.9566	0.9579	0.1711	<0.0001	0.0030*	0.0499*
1 Day - 3 Days	0.7210**	0.0011	0.7544	0.0209	0.3120	0.2790*	0.0009	<0.0001	0.0104*
3 Days - 5 Days	0.6684	0.1050**	0.0006*	0.0074	0.0070*	0.0118	0.3950	0.0001	0.0499*
5 Days - 7 Days	0.7210*	0.6968	0.2453	0.8827	0.7980*	0.0021	0.3213	0.2790*	0.7210*
7 Days - 14 Days	0.7378	0.2076	0.0023	0.4520	0.5740*	0.3820*	0.8101	0.4220*	1.0000
14 Days - 28 Days	0.5050**	0.0104*	0.8780*	0.0207*	0.1050*	0.7210*	0.0161	0.7210*	1.0000
28 Days - 42 Days	0.3902*	0.3820*	0.4370	0.2790*	0.1300*	0.7621*	0.4062	0.7210*	1.0000

Table 3.3: T test analysis of volumetric results between concurrent time points.

* = Normality Test Failed; Mann-Whitney Rank Sum Test ran.

** = Equal Variance Test Failed; Mann-Whitney Rank Sum Test ran.

Injury Time		Adhesion		Peritoneal Tissue Generation		Uterine Horn Tissue Generation		Free Floating Tissue Generation		Total Tissue Generation		Percentage	
		Fibrin	Collagen	Fibrin	Collagen	Fibrin	Collagen	Fibrin	Collagen	Fibrin	Collagen	Fibrin	Collagen
30 SECS	**Mean**	**0.00**	**0.00**	**0.77**	**0.00**	**0.18**	**0.00**	**0.00**	**0.00**	**0.95**	**0.00**	**100.00**	**0.00**
	STD	0.00	0.00	0.92	0.00	0.31	0.00	0.00	0.00	1.16	0.00	0.00	0.00
	SEM	0.00	0.00	0.33	0.00	0.11	0.00	0.00	0.00	0.41	0.00	0.00	0.00
	CV%	N/A	N/A	42.41	N/A	61.07	N/A	N/A	N/A	43.23	N/A	0.00	N/A
1 MIN	**Mean**	**0.00**	**0.00**	**0.39**	**0.00**	**0.12**	**0.00**	**0.00**	**0.00**	**0.51**	**0.00**	**100.00**	**0.00**
	STD	0.00	0.00	0.16	0.00	0.15	0.00	0.00	0.00	0.15	0.00	0.00	0.00
	SEM	0.00	0.00	0.06	0.00	0.05	0.00	0.00	0.00	0.05	0.00	0.00	0.00
	CV%	N/A	N/A	14.54	N/A	42.16	N/A	N/A	N/A	10.66	N/A	0.00	N/A
3 MINS	**Mean**	**0.00**	**0.00**	**1.79**	**0.00**	**0.12**	**0.00**	**0.00**	**0.00**	**1.92**	**0.00**	**100.00**	**0.00**
	STD	0.00	0.00	1.12	0.00	0.13	0.00	0.00	0.00	1.15	0.00	0.00	0.00
	SEM	0.00	0.00	0.40	0.00	0.05	0.00	0.00	0.00	0.41	0.00	0.00	0.00
	CV%	N/A	N/A	22.09	N/A	37.42	N/A	N/A	N/A	21.26	N/A	0.00	N/A
5 MINS	**Mean**	**0.00**	**0.00**	**2.57**	**0.00**	**0.28**	**0.00**	**0.00**	**0.00**	**2.85**	**0.00**	**100.00**	**0.00**
	STD	0.00	0.00	2.41	0.00	0.41	0.00	0.00	0.00	2.70	0.00	0.00	0.00
	SEM	0.00	0.00	0.85	0.00	0.15	0.00	0.00	0.00	0.95	0.00	0.00	0.00
	CV%	N/A	N/A	33.16	N/A	51.88	N/A	N/A	N/A	33.47	N/A	0.00	N/A
10 MINS	**Mean**	**0.00**	**0.00**	**1.29**	**0.00**	**0.48**	**0.00**	**0.00**	**0.00**	**1.77**	**0.00**	**100.00**	**0.00**
	STD	0.00	0.00	0.56	0.00	0.87	0.00	0.00	0.00	0.81	0.00	0.00	0.00
	SEM	0.00	0.00	0.20	0.00	0.31	0.00	0.00	0.00	0.28	0.00	0.00	0.00
	CV%	N/A	N/A	15.44	N/A	63.79	N/A	N/A	N/A	16.04	N/A	0.00	N/A
30 MINS	**Mean**	**0.00**	**0.00**	**1.19**	**0.00**	**0.13**	**0.00**	**0.00**	**0.00**	**1.32**	**0.00**	**100.00**	**0.00**
	STD	0.00	0.00	1.25	0.00	0.23	0.00	0.00	0.00	1.32	0.00	0.00	0.00
	SEM	0.00	0.00	0.44	0.00	0.08	0.00	0.00	0.00	0.47	0.00	0.00	0.00
	CV%	N/A	N/A	37.14	N/A	61.13	N/A	N/A	N/A	35.45	N/A	0.00	N/A
1 HR	**Mean**	**0.10**	**0.00**	**1.00**	**0.00**	**0.06**	**0.00**	**0.00**	**0.00**	**1.17**	**0.00**	**100.00**	**0.00**
	STD	0.18	0.00	0.71	0.00	0.09	0.00	0.00	0.00	0.70	0.00	0.00	0.00
	SEM	0.06	0.00	0.25	0.00	0.03	0.00	0.00	0.00	0.25	0.00	0.00	0.00
	CV%	62.02	N/A	25.09	N/A	54.25	N/A	N/A	N/A	21.10	N/A	0.00	N/A
4 HRS	**Mean**	**4.46**	**0.00**	**1.69**	**0.00**	**0.97**	**0.00**	**0.00**	**0.00**	**7.12**	**0.00**	**100.00**	**0.00**
	STD	7.00	0.00	1.28	0.00	1.20	0.00	0.00	0.00	6.66	0.00	0.00	0.00
	SEM	2.48	0.00	0.45	0.00	0.43	0.00	0.00	0.00	2.35	0.00	0.00	0.00
	CV%	55.55	N/A	26.76	N/A	43.67	N/A	N/A	N/A	33.06	N/A	0.00	N/A
8 HRS	**Mean**	**11.45**	**0.00**	**1.93**	**0.00**	**1.46**	**0.00**	**0.00**	**0.00**	**14.84**	**0.00**	**100.00**	**0.00**
	STD	10.22	0.00	1.47	0.00	1.18	0.00	0.00	0.00	10.90	0.00	0.00	0.00
	SEM	3.61	0.00	0.52	0.00	0.42	0.00	0.00	0.00	3.85	0.00	0.00	0.00
	CV%	31.55	N/A	26.93	N/A	28.52	N/A	N/A	N/A	25.95	N/A	0.00	N/A
16 HRS	**Mean**	**12.24**	**0.00**	**1.44**	**0.00**	**1.34**	**0.00**	**0.00**	**0.00**	**15.03**	**0.00**	**100.00**	**0.00**
	STD	6.04	0.00	1.02	0.00	3.16	0.00	0.00	0.00	6.09	0.00	0.00	0.00
	SEM	2.14	0.00	0.36	0.00	1.12	0.00	0.00	0.00	2.15	0.00	0.00	0.00
	CV%	17.45	N/A	24.90	N/A	83.08	N/A	N/A	N/A	14.34	N/A	0.00	N/A
1 DAY	**Mean**	**10.30**	**0.01**	**1.91**	**0.00**	**0.80**	**0.00**	**0.00**	**0.00**	**13.01**	**0.01**	**99.93**	**0.07**
	STD	5.10	0.02	1.25	0.01	0.51	0.00	0.00	0.00	4.61	0.02	0.14	0.14
	SEM	1.80	0.01	0.44	0.00	0.18	0.00	0.00	0.00	1.63	0.01	0.05	0.05
	CV%	17.50	100.00	23.21	100.00	22.48	N/A	N/A	N/A	12.54	76.20	0.05	74.69
3 DAYS	**Mean**	**22.59**	**1.00**	**13.12**	**1.15**	**1.97**	**0.01**	**0.24**	**0.00**	**37.92**	**2.16**	**91.80**	**8.20**
	STD	29.91	0.90	10.17	1.43	2.30	0.02	0.51	0.00	30.09	2.02	9.63	9.63
	SEM	10.57	0.32	3.59	0.51	0.81	0.01	0.18	0.00	10.64	0.72	3.41	3.41
	CV%	46.80	31.51	27.39	44.16	41.43	100.00	74.62	N/A	28.05	33.14	3.71	41.57
5 DAYS	**Mean**	**4.78**	**27.60**	**1.18**	**29.89**	**0.81**	**13.54**	**0.00**	**0.00**	**6.78**	**71.02**	**9.57**	**90.43**
	STD	8.19	26.66	1.45	15.32	1.60	5.17	0.00	0.00	7.35	30.26	9.66	9.66
	SEM	2.89	9.43	0.51	5.42	0.57	1.83	0.00	0.00	2.60	10.70	3.41	3.41
	CV%	60.49	34.16	43.25	18.12	69.81	13.52	N/A	N/A	38.36	15.06	35.67	3.78
7 DAYS	**Mean**	**3.51**	**37.04**	**1.04**	**55.04**	**0.52**	**18.84**	**0.00**	**0.00**	**5.08**	**110.92**	**3.99**	**96.01**
	STD	5.25	37.81	1.79	33.11	1.33	11.48	0.00	0.00	6.01	43.23	5.04	5.04
	SEM	1.85	13.37	0.63	11.71	0.47	4.06	0.00	0.00	2.12	15.29	1.78	1.78
	CV%	52.84	36.10	60.70	21.27	90.31	21.54	N/A	N/A	41.81	13.78	44.60	1.85
14 DAYS	**Mean**	**0.92**	**38.40**	**0.33**	**107.70**	**0.03**	**14.36**	**0.00**	**0.00**	**1.28**	**160.46**	**0.77**	**99.23**
	STD	1.52	34.60	0.54	47.81	0.07	8.73	0.00	0.00	1.67	54.79	0.85	0.85
	SEM	0.54	12.23	0.19	16.90	0.03	3.09	0.00	0.00	0.59	19.37	0.30	0.30
	CV%	58.21	31.86	57.14	15.69	100.00	21.49	N/A	N/A	46.17	12.07	39.18	0.30
28 DAYS	**Mean**	**0.00**	**20.17**	**0.00**	**53.81**	**0.00**	**10.99**	**0.00**	**0.00**	**0.00**	**84.98**	**0.00**	**100.00**
	STD	0.00	14.26	0.00	100.87	0.00	16.54	0.00	0.00	0.00	99.82	0.00	0.00
	SEM	0.00	5.04	0.00	35.66	0.00	5.85	0.00	0.00	0.00	35.29	0.00	0.00
	CV%	N/A	24.99	N/A	66.27	N/A	53.21	N/A	N/A	N/A	41.53	N/A	0.00
42 DAYS	**Mean**	**0.00**	**31.03**	**0.00**	**26.91**	**0.00**	**9.50**	**0.00**	**0.00**	**0.00**	**67.44**	**0.00**	**100.00**
	STD	0.00	28.34	0.00	17.42	0.00	4.39	0.00	0.00	0.00	22.94	0.00	0.00
	SEM	0.00	10.02	0.00	6.16	0.00	1.55	0.00	0.00	0.00	8.11	0.00	0.00
	CV%	N/A	32.29	N/A	22.88	N/A	16.33	N/A	N/A	N/A	12.03	N/A	0.00

Table 3.4: Mean volumes of fibrin and collagen, together with SDs, SEMs and CV%.

Injury Time	P VALUE			
	Total Tissue Generation			
	Volume		Percentage	
	Fibrin	Collagen	Fibrin	Collagen
30 Seconds - 1 Minute	0.7980	1.0000	1.0000	1.0000
1 Minute - 3 Minutes	0.0104**	1.0000	1.0000	1.0000
3 Minutes - 5 Minutes	0.3816*	1.0000	1.0000	1.0000
5 Minutes - 10 Minutes	0.7210*	1.0000	1.0000	1.0000
10 Minutes - 30 Minutes	0.4193	1.0000	1.0000	1.0000
30 MInutes - 1 Hour	0.7841	1.0000	1.0000	1.0000
1 Hour - 4 Hours	0.0030*	1.0000	1.0000	1.0000
4 Hours - 8 Hours	0.1104*	1.0000	1.0000	1.0000
8 Hours - 16 Hours	0.9450	1.0000	1.0000	1.0000
16 Hours - 24 Hours	0.4679	0.4420*	0.4420*	0.4420*
24 Hours - 3 Days	0.0019*	0.0030*	0.00295*	0.00295*
3 Days - 5 Days	0.0011*	0.0002*	<0.0001	<0.0001
5 Days - 7 Days	0.5740*	0.0506	0.1300*	0.1300*
7 Days - 14 Days	0.1067	0.0644	0.5050*	0.5050*
14 Days - 28 Days	0.0104*	0.0207*	0.0104*	0.0104*
28 Days - 42 Days	1.0000	0.4420*	1.0000	1.0000

Table 3.5: T test analysis of fibrin and collagen mean volumes and percentages between control and treated cases.

* = Normality Test Failed; Mann-Whitney Rank Sum Test ran.

** = Equal Variance Test Failed; Mann-Whitney Rank Sum Test ran.

Injury Time	P VALUE		
	PSA against PTG	PSA against UTG	PTG against UTG
1 Hour	-	-	-
4 Hours	-	-	-
8 Hours	-	-	-
16 Hours	-	-	-
24 Hours	0.959*	0.721*	0.721*
3 Days	0.959*	0.00373*	0.000311*
5 Days	0.222*	0.0653	0.382*
7 Days	0.0451*	0.0109*	0.798*
14 Days	0.142*	0.0593*	0.328*
28 Days	-	-	-
42 Days	-	-	-

Table 3.6: T test analysis comparing fibrin and collagen mean percentages within tissue zones.

* = Normality Test Failed; Mann-Whitney Rank Sum Test ran.

Injury Time	P VALUE			
	Total Tissue Generation			
	Volume		Percentage	
	Fibrin	Collagen	Fibrin	Collagen
1 Hour	0.3622	1.0000	1.0000	1.0000
4 Hours	-	-	-	-
8 Hours	-	-	-	-
16 Hours	-	-	-	-
24 Hours	-	-	-	-
3 Days	-	-	-	-
5 Days	0.3797	0.0095	0.9340	0.9340
7 Days	0.0531	0.0635	0.0771	0.0771
14 Days	0.7983	0.3599	0.7927	0.7927
28 Days	1.0000	0.0714**	1.0000	1.0000
42 Days	1.0000	0.0199	1.0000	1.0000

Table 3.7: T test analysis of fibrin and collagen composition within adhered and non adhered control cases.

* = Normality Test Failed; Mann-Whitney Rank Sum Test ran.

** = Equal Variance Test Failed; Mann-Whitney Rank Sum Test ran.

Animal No:	Injury Time		PSA	Tissue Generation				Damage Zone		Peritoneal Reaction Zone	Peritoneal Cell Conc	U/Horn Haem.
				Peritoneal	Uterine Horn	Free Floating	Total	Peritoneal	Uterine Horn			
Adhered Cases	1 Hour	**Mean**	**6.95**	**17.95**	**2.43**	**0.00**	**27.33**	**13.09**	**12.71**	**79.36**	**0.00**	**11.77**
		STD	3.92	2.48	3.44	0.00	4.87	9.35	6.52	6.86	0.00	3.97
		SEM	2.77	1.76	2.43	0.00	3.44	6.61	4.61	4.85	0.00	2.81
		CV%	39.85	9.78	100.00	N/A	12.60	50.52	36.27	6.11	N/A	23.88
Non Adhered Cases		**Mean**	**0.00**	**16.73**	**0.11**	**0.47**	**17.31**	**14.72**	**3.72**	**72.15**	**0.00**	**10.38**
		STD	0.00	19.24	0.27	0.88	19.63	9.61	5.69	35.19	0.00	4.73
		SEM	0.00	7.86	0.11	0.36	8.02	3.92	2.32	14.37	0.00	1.93
		CV%	N/A	46.95	100.00	75.83	46.30	26.65	62.53	19.91	N/A	18.61
Adhered Cases	4 Hours	**Mean**	**7.62**	**15.40**	**10.15**	**0.16**	**33.33**	**7.67**	**10.94**	**100.20**	**0.16**	**10.12**
		STD	8.05	6.51	9.02	0.32	11.21	0.73	9.85	19.53	0.33	5.99
		SEM	4.02	3.25	4.51	0.16	5.61	0.36	4.93	9.77	0.16	2.99
		CV%	52.80	21.12	44.42	100.00	16.82	4.73	45.02	9.75	100.00	29.59
Non Adhered Cases		**Mean**	**0.00**	**21.75**	**3.07**	**0.17**	**24.99**	**10.29**	**5.74**	**90.24**	**0.61**	**10.36**
		STD	0.00	5.79	2.64	0.35	7.42	4.42	3.99	31.49	0.82	4.28
		SEM	0.00	2.90	1.32	0.17	3.71	2.21	1.99	15.75	0.41	2.14
		CV%	N/A	13.31	43.08	100.00	14.85	21.47	34.74	17.45	67.49	20.68
	8 Hours	**N/A (all cases adhered)**										
	16 Hours	**N/A (all cases adhered)**										
	1 Day	**N/A (all cases adhered)**										
Adhered Cases	3 Days	**Mean**	**48.11**	**58.71**	**7.46**	**2.74**	**117.02**	**19.67**	**5.85**	**216.68**	**33.05**	**6.26**
		STD	41.86	18.48	9.01	7.26	54.59	8.13	5.82	63.62	6.64	13.43
		SEM	15.82	6.99	3.41	2.74	20.63	3.07	2.20	24.05	2.51	5.08
		CV%	32.89	11.90	45.70	100.00	17.63	15.61	37.57	11.10	7.59	81.15
Non Adhered Cases		**Mean**	**0.00**	**93.22**	**1.64**	**0.00**	**94.85**	**21.26**	**1.64**	**233.05**	**44.97**	**0.82**
		STD	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
		SEM	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
		CV%	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Adhered Cases	5 Days	**Mean**	**84.89**	**144.55**	**43.69**	**0.64**	**273.78**	**59.10**	**20.65**	**306.30**	**5.49**	**0.00**
		STD	47.78	78.86	28.38	0.91	68.49	32.29	9.96	110.92	9.32	0.00
		SEM	21.37	35.27	12.69	0.41	30.63	14.44	4.46	49.61	4.17	0.00
		CV%	25.17	24.40	29.05	63.25	11.19	24.43	21.57	16.20	76.00	N/A
Non Adhered Cases		**Mean**	**0.00**	**94.71**	**56.62**	**0.45**	**151.78**	**33.89**	**16.58**	**176.38**	**15.23**	**0.22**
		STD	0.00	72.65	7.89	0.39	79.61	23.75	6.72	46.69	14.48	0.39
		SEM	0.00	41.95	4.56	0.23	45.96	13.71	3.88	26.96	8.36	0.22
		CV%	N/A	44.29	8.05	50.36	30.28	40.46	23.38	15.28	54.88	100.00
Adhered Cases	7 Days	**Mean**	**91.88**	**149.62**	**36.65**	**0.00**	**278.14**	**64.65**	**4.23**	**208.04**	**3.25**	**0.00**
		STD	17.09	110.16	21.48	0.00	127.22	42.90	2.69	124.51	7.27	0.00
		SEM	7.64	49.26	9.61	0.00	56.89	19.18	1.20	55.68	3.25	0.00
		CV%	8.32	32.93	26.22	N/A	20.45	29.67	28.48	26.76	100.00	N/A
Non Adhered Cases		**Mean**	**0.00**	**129.77**	**35.76**	**0.00**	**165.53**	**31.76**	**0.98**	**191.38**	**0.69**	**0.00**
		STD	0.00	48.80	9.48	0.00	56.67	14.82	1.70	92.50	1.20	0.00
		SEM	0.00	28.17	5.48	0.00	32.72	8.56	0.98	53.40	0.69	0.00
		CV%	N/A	21.71	15.31	N/A	19.77	26.95	100.00	27.90	100.00	N/A
Adhered Cases	14 Days	**Mean**	**65.18**	**278.72**	**13.44**	**0.11**	**357.46**	**82.64**	**3.41**	**247.64**	**0.00**	**0.00**
		STD	46.88	206.33	9.43	0.28	199.19	67.49	5.11	42.99	0.00	0.00
		SEM	19.14	84.24	3.85	0.11	81.32	27.55	2.09	17.55	0.00	0.00
		CV%	29.36	30.22	28.64	100.00	22.75	33.34	61.24	7.09	N/A	N/A
Non Adhered Cases		**Mean**	**0.00**	**121.89**	**2.44**	**0.00**	**124.33**	**21.24**	**0.00**	**109.16**	**0.00**	**0.00**
		STD	0.00	11.27	1.56	0.00	12.83	9.19	0.00	5.79	0.00	0.00
		SEM	0.00	7.97	1.10	0.00	9.07	6.50	0.00	4.09	0.00	0.00
		CV%	N/A	6.54	45.09	N/A	7.29	30.60	N/A	3.75	N/A	N/A
Adhered Cases	28 Days	**Mean**	**29.49**	**45.94**	**15.25**	**0.00**	**90.67**	**18.38**	**1.67**	**129.48**	**0.00**	**0.00**
		STD	17.43	25.40	27.55	0.00	43.07	12.10	1.84	24.51	0.00	0.00
		SEM	6.59	9.60	10.41	0.00	16.28	4.57	0.69	9.26	0.00	0.00
		CV%	22.34	20.90	68.29	N/A	17.95	24.88	41.46	7.15	N/A	N/A
Non Adhered Cases		**Mean**	**0.00**	**547.50**	**47.50**	**0.00**	**595.00**	**167.50**	**4.38**	**190.63**	**3.13**	**0.00**
		STD	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
		SEM	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
		CV%	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Adhered Cases	42 Days	**Mean**	**43.47**	**73.61**	**28.29**	**0.00**	**145.37**	**15.94**	**1.96**	**150.18**	**0.00**	**0.00**
		STD	32.92	50.45	8.06	0.00	59.32	15.12	1.84	23.70	0.00	0.00
		SEM	12.44	19.07	3.05	0.00	22.42	5.71	0.70	8.96	0.00	0.00
		CV%	28.62	25.90	10.77	N/A	15.42	35.85	35.40	5.97	N/A	N/A
Non Adhered Cases		**Mean**	**0.00**	**41.41**	**21.88**	**0.00**	**63.28**	**1.56**	**0.00**	**120.31**	**0.00**	**0.00**
		STD	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
		SEM	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
		CV%	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 3.8: Volumetric results of tissue zones for adhered and non-adhered cases.

Injury Time	P VALUE								
	PSA	Tissue Generation			Damage Zone		Peritoneal Reaction Zone	Peritoneal Cell Concentration	Uterine Horn Haemorrhage
		Peritoneal	Uterine Horn	Total	Peritoneal	Uterine Horn			
1 Hour	0.0952*	0.0952**	0.3810*	0.0004	0.8570*	0.1900*	0.2036	N/A	0.9774
4 Hours	0.1070	0.1956	0.4860**	0.2609	0.4860**	0.6860**	0.6102	0.3567	0.9501
8 Hours	N/A (all cases Adhered)								
16 Hours	N/A (all cases Adhered)								
1 Day	N/A (all cases Adhered)								
3 Days	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
5 Days	0.0246	0.4087	0.7860**	0.0605	0.2895	0.5597	0.1087	0.2826	0.5710*
7 Days	0.0001	0.7828	0.9495	0.2066	0.2581	0.1146	0.8490	1.0000*	N/A
14 Days	0.1114	0.3473	0.0714*	0.1676	0.2689	0.4055	0.0050	N/A	N/A
28 Days	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
42 Days	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 3.9: T test analysis of volumetric results of tissue zones for adhered and non-adhered cases.

Injury Time		POST SURGICAL ADHESION						PERITONEAL TISSUE GENERATION						UTERINE HORN TISSUE GENERATION					
		Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.
1 Hour	Mean	N/A	N/A	N/A	N/A	N/A	N/A	78474.20	36144.70	37286.36	0.00	6867.31	158772.56	179351.81	24086.30	32639.26	395.62	9828.81	246301.80
	%	0.00	0.00	0.00	0.00	N/A	0.00	49.43	22.77	23.48	0.00	4.33	100.00	72.82	9.78	13.25	0.16	3.99	100.00
	STD	N/A	N/A	N/A	N/A	N/A	N/A	59056.66	32631.19	61644.53	0.00	12618.33	49436.51	123403.39	22010.13	30487.15	734.54	16446.63	88164.11
	SEM	0.00	0.00	0.00	0.00	N/A	0.00	20879.68	11536.87	21794.63	0.00	4461.25	17478.45	43629.69	7781.76	10778.83	259.70	5814.76	31170.72
	CV%	N/A	N/A	N/A	N/A	N/A	N/A	26.61	31.92	58.45	N/A	64.96	11.01	24.33	32.31	33.02	65.64	59.16	12.66
4 Hours	Mean	58724.28	191069.96	47695.47	2386.83	5473.25	305349.79	60890.66	175498.16	12054.77	798.06	2293.43	251535.07	97359.42	219774.73	27453.57	0.00	4338.04	348925.76
	%	19.23	62.57	15.62	0.78	1.79	100.00	24.21	69.77	4.79	0.32	0.91	100.00	27.90	62.99	7.87	0.00	1.24	100.00
	STD	29584.93	88046.10	38964.33	3315.76	4179.94	97806.56	32533.81	72163.41	11651.21	854.84	2216.81	89041.91	57760.69	69533.45	43748.47	0.00	5114.94	78710.34
	SEM	17080.87	50833.44	22496.06	1914.35	2413.29	56468.64	11502.44	25513.62	4119.33	302.23	783.76	31481.07	21831.49	26281.17	16535.37	0.00	1933.27	29749.71
	CV%	29.09	26.60	47.17	80.20	44.09	18.49	18.89	14.54	34.17	37.87	34.17	12.52	22.42	11.96	60.23	#DIV/0!	44.57	8.53
8 Hours	Mean	62440.69	264448.99	11737.60	320.83	2980.44	341928.55	59682.38	224140.95	16341.45	0.00	2552.13	302716.91	96729.48	286246.48	9075.16	1258.93	2826.51	396136.56
	%	18.26	77.34	3.43	0.09	0.87	100.00	19.72	74.04	5.40	0.00	0.84	100.00	24.42	72.26	2.29	0.32	0.71	100.00
	STD	38747.40	79019.48	8269.00	692.56	3054.84	79559.02	37192.59	162460.66	5937.80	0.00	4586.90	163338.41	68926.96	159716.04	9113.44	1436.50	3335.56	216073.53
	SEM	13699.27	27937.60	2923.53	244.86	1080.05	28128.36	16633.03	72654.62	2655.46	0.00	2051.32	73047.16	34463.48	79858.02	4556.72	718.25	1667.78	108036.77
	CV%	21.94	10.56	24.91	76.32	36.24	8.23	27.87	32.41	16.25	N/A	80.38	24.13	35.63	27.90	50.21	57.05	59.00	27.27
16 Hours	Mean	106844.62	366206.34	25679.63	5254.49	4578.61	508563.70	181558.11	520639.79	30524.84	0.00	0.00	732722.74	139525.77	361160.10	49186.75	16264.94	5486.97	571624.53
	%	21.01	72.01	5.05	1.03	0.90	100.00	24.78	71.06	4.17	0.00	0.00	100.00	24.41	63.18	8.60	2.85	0.96	100.00
	STD	37991.69	82242.07	15749.49	5904.01	4306.06	96224.16	103684.17	260135.07	10263.15	0.00	0.00	338849.75	42124.33	16905.16	54595.35	8036.88	7759.75	45172.80
	SEM	13432.09	29076.96	5568.28	2087.38	1522.42	34020.38	59862.09	150189.05	5925.43	0.00	0.00	195634.99	29786.40	11953.75	38604.74	5682.93	5486.97	31941.99
	CV%	12.57	7.94	21.68	39.73	33.25	6.69	32.97	28.85	19.41	N/A	N/A	26.70	21.35	3.31	78.49	34.94	100.00	5.59
1 Day	Mean	85890.32	300495.90	17686.15	2205.25	3190.53	409468.15	65791.71	260303.54	9751.16	1322.14	974.83	338143.38	93871.13	163610.19	19086.52	2777.78	462.96	279808.59
	%	20.98	73.39	4.32	0.54	0.78	100.00	19.46	76.98	2.88	0.39	0.29	100.00	33.55	58.47	6.82	0.99	0.17	100.00
	STD	15732.22	124564.93	19496.79	3146.53	2735.50	148663.48	38288.76	147655.41	8085.76	1832.19	1541.47	170659.62	63714.57	104233.70	38564.06	6211.30	1035.22	93145.80
	SEM	5562.18	44040.36	6893.16	1112.47	967.15	52560.48	17123.25	66033.51	3616.06	819.38	689.37	76321.30	28494.02	46614.73	17246.37	2777.78	462.96	41656.07
	CV%	6.48	14.66	38.97	50.45	30.31	12.84	26.03	25.37	37.08	61.97	70.72	22.57	30.35	28.49	90.36	100.00	100.00	14.89
3 Days	Mean	52636.00	25201.19	115539.96	107856.64	7065.35	308299.14	47646.60	22182.57	108520.72	137440.23	4548.55	320338.68	13127.26	31049.85	76040.94	169189.46	2134.03	291541.54
	%	17.07	8.17	37.48	34.98	2.29	100.00	14.87	6.92	33.88	42.90	1.42	100.00	4.50	10.65	26.08	58.03	0.73	100.00
	STD	53383.31	20593.67	77194.83	88611.11	5418.02	153752.50	61822.09	25101.11	71797.89	71046.05	6082.97	130203.80	9644.79	57020.11	81060.33	116480.26	2781.39	191476.29
	SEM	23873.74	9209.77	34522.58	39628.10	2423.01	68760.21	25238.76	10247.48	29311.36	29004.43	2483.36	53155.48	4822.40	28510.05	40530.16	58240.13	1390.70	95738.15
	CV%	45.36	36.54	29.88	36.74	34.29	22.30	52.97	46.20	27.01	21.10	54.60	16.59	36.74	91.82	53.30	34.42	65.17	32.84
5 Days	Mean	42688.59	23550.28	156747.67	425713.16	1384.56	650084.25	22295.76	5451.42	80232.84	270746.18	839.34	379565.53	25575.51	2884.62	99617.00	383476.87	1744.63	513298.63
	%	6.57	3.62	24.11	65.49	0.21	100.00	5.87	1.44	21.14	71.33	0.22	100.00	4.98	0.56	19.41	74.71	0.34	100.00
	STD	38891.30	27883.62	105068.49	173573.36	162105.25	332798.08	28300.81	3593.58	24028.85	123055.76	1314.70	140287.69	40847.32	4310.76	56936.80	213458.41	1956.75	286616.28
	SEM	15877.31	11383.44	42894.03	70861.03	66179.19	135864.25	11553.76	1467.07	9809.74	50237.30	536.72	57272.21	16675.85	1759.86	23244.35	87144.03	798.84	117010.61
	CV%	37.19	48.34	27.37	16.65	4779.79	20.90	51.82	26.91	12.23	18.56	63.95	15.09	65.20	61.01	23.33	22.72	45.79	22.80
7 Days	Mean	12834.71	4187.38	57339.77	225933.41	489.03	300784.30	8026.69	6607.44	76943.46	241357.80	1198.76	334134.15	21557.57	4588.36	52379.58	273338.09	3149.54	355013.14
	%	4.27	1.39	19.06	75.11	0.16	100.00	2.40	1.98	23.03	72.23	0.36	100.00	6.07	1.29	14.75	76.99	0.89	100.00
	STD	30009.80	2991.57	59556.36	94871.06	763.60	177815.65	18900.87	7933.92	82100.00	84291.74	1495.08	100074.89	53515.11	4779.87	54117.12	90677.53	5266.26	77734.96
	SEM	12251.45	1221.30	24313.78	38730.95	311.74	72592.94	7143.86	2998.74	31030.88	31859.28	565.09	37824.75	20226.81	1806.62	20454.35	34272.88	1990.46	29381.05
	CV%	95.46	29.17	42.40	17.14	63.75	24.13	89.00	45.38	40.33	13.20	47.14	11.32	93.83	39.37	39.05	12.54	63.20	8.28
14 Days	Mean	14354.00	1250.14	28592.79	222256.36	1233.15	267686.43	9158.45	1386.43	16875.42	280601.32	797.03	308818.65	9847.15	1006.20	24936.27	313608.17	3363.41	352422.44
	%	5.36	0.47	10.68	83.03	0.46	100.00	2.97	0.45	5.46	90.86	0.26	100.00	2.79	0.29	7.08	88.99	0.95	100.10
	STD	34036.55	1654.49	26767.91	94766.06	1248.93	114490.71	22354.85	1486.19	14360.16	84198.16	1168.46	90345.80	22436.86	1749.22	19660.91	84540.32	6838.95	108230.73
	SEM	13895.36	675.44	10927.95	38688.08	509.87	46740.64	8449.34	561.73	5427.63	31823.91	441.64	34147.50	9159.81	714.11	8026.53	34513.44	2791.99	44185.01
	CV%	96.80	54.03	38.22	17.41	41.35	17.46	92.26	40.52	32.16	11.34	55.41	11.06	93.02	70.97	32.19	11.01	83.01	12.54
28 Days	Mean	10447.14	1416.12	24006.75	313167.74	1264.56	350302.31	142.45	877.93	13449.23	193832.96	2502.07	210804.64	0.00	131.45	10272.67	291785.52	1287.33	303476.98
	%	2.98	0.40	6.85	89.40	0.36	100.00	0.07	0.42	6.38	91.95	1.19	100.00	0.00	0.04	3.38	96.15	0.42	100.00
	STD	26785.88	2103.84	30055.90	76567.48	1656.20	89196.96	402.91	1511.32	9088.63	48134.72	4498.52	48543.89	0.00	347.79	8208.02	96058.23	2013.44	95267.67
	SEM	10124.11	795.17	11360.06	28939.79	625.98	33713.28	142.45	534.33	3213.32	17018.19	1590.47	17162.86	0.00	131.45	3102.34	36306.60	761.01	36007.79
	CV%	96.91	56.15	47.32	9.24	49.50	9.62	100.00	60.86	23.89	8.78	63.57	8.14	#DIV/0!	100.00	30.20	12.44	59.12	11.87
42 Days	Mean	7993.72	536.77	10655.80	219552.50	967.76	239706.55	31498.67	2416.88	24388.87	226737.24	2155.59	287197.25	18504.61	6464.95	22258.23	284724.38	1667.98	333620.15
	%	3.33	0.22	4.45	91.59	0.40	100.00	10.97	0.84	8.49	78.95	0.75	100.00	5.55	1.94	6.67	85.34	0.50	100.00
	STD	14344.32	1314.81	16055.47	179726.62	1550.33	198170.35	34011.42	3702.61	13723.04	133048.61	4180.04	155283.25	20749.61	7872.12	15912.57	109435.15	2711.72	135245.17
	SEM	5856.04	536.77	6554.62	73373.08	632.92	80902.71	12855.11	1399.46	5186.82	50287.65	1579.91	58691.55	8470.99	3213.78	6496.28	44676.71	1107.05	55213.61
	CV%	73.26	100.00	61.51	33.42	65.40	33.75	40.81	57.90	21.27	22.18	73.29	20.44	45.78	49.71	29.19	15.69	66.37	16.55

Table 3.10: Mean number densities of cell types within PSAs, PTG and UTG from 1hour to 42 days, together with mean percentages, SDs, SEMs and CV%.

Injury Time	P Value Post Surgical Adhesion						P Value Peritoneal Tissue Generation						P Value Uterine Horn Tissue Generation					
	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.
1-4 Hours	-	-	-	-	-	-	0.2611	0.0005	0.6940*	0.1210*	0.7790*	0.0127	0.0366	0.0006**	0.5350*	0.3830*	0.9683	0.0644
4-8 Hours	0.8850	0.2143	0.0249	0.0899*	0.2986	0.5359	0.9518	0.4685	0.1270*	0.0647	0.6220*	0.4752	0.9868	0.2452	0.7880*	0.2300*	0.6480*	0.4896
8-16 Hours	0.0363	0.0243	0.0437	0.0281*	0.4063	0.0020	0.0714**	0.1430*	0.0443	1.0000	0.3930*	0.0474	0.4396	0.8000**	0.2670**	0.1330**	0.5691	0.1330**
16 Hours-1 Day	0.3820**	0.2335	0.1950*	0.3820*	0.4543	0.1358	0.1430**	0.1136	0.0184	0.2717	0.3297	0.0655	0.4050	0.0529	0.3810*	0.1900*	0.5710*	0.0096
1-3 Days	0.1410	0.0005	0.0062**	0.0031*	0.2840**	0.2633	0.5834	0.0087*	0.0043**	0.0043*	0.2361	0.8485	0.1110**	0.0578	0.1110*	0.0141	0.2491	0.9066
3-5 Days	0.7312	0.5370*	0.3911	0.0007	0.0173*	0.0115	0.3826	0.0649*	0.9370**	0.0444	0.1750	0.4660	0.9140*	0.7620*	0.6006	0.1076	1.0000*	0.2150
5-7 Days	0.0411*	0.1320*	0.0260*	0.0085	0.8180*	0.0089	0.1010*	0.9450*	0.2950*	0.6206	0.7310*	0.5104	0.2340*	0.5168	0.1537	0.2382	0.8360*	0.1858
7-14 Days	0.9370*	0.0616	0.5890*	0.9478	0.3940*	0.7095	0.9020*	0.0973*	0.0262*	0.4560*	0.5857	0.6283	0.6280*	0.1116	0.2662	0.4279	0.7310*	0.9609
14-28 Days	0.6280*	0.9450*	0.7310*	0.0818	0.9450*	0.1714	0.2810*	0.5360*	0.5849	0.0269	0.9550*	0.0193	0.3660*	0.5340*	0.0980	0.6748	1.0000*	0.4040
28-42 Days	0.5340*	0.4450*	0.5340*	0.4544	0.8360*	0.4412	0.0939*	0.6940*	0.0881	0.7790**	0.7790*	0.7790**	0.1380*	0.2340*	0.1082	0.9036	1.0000*	0.6472

Table 3.11: T test analysis comparing number densities of cell types with PSAs, PTG and UTG between concurrent time points.

Injury Time	P Value PSA vs PTG						P Value PSA vs UTG						P Value PTG vs UTG					
	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.
1 Hour	-	-	-	-	-	-	-	-	-	-	-	-	0.0235	0.1967	0.3202	0.3830*	0.3830*	0.0101
4 Hours	0.9223	0.7690	0.0848*	0.7760**	0.1249	0.4054	0.3144	0.5923	0.2670*	0.1170*	0.7456	0.4732	0.1890**	0.2495	0.8670*	0.1210*	0.3220	0.0441
8 Hours	0.9015	0.5562	0.3050	0.5240*	0.8422	0.5690	0.2604	0.5562	0.6254	0.3680*	0.9392	0.4610**	0.3001	0.5365	0.1986	0.2860*	0.9050*	0.3985
16 Hours	0.3760**	0.7760**	0.6382	0.0848*	0.1086	0.1004	0.3146	0.9361	0.7110**	0.0552	0.8195	0.4083	0.8000**	0.4715	0.5753	0.0312	0.2722	0.5705
1 Day	0.2840**	0.6077	0.4350*	0.6220*	0.1296	0.4424	0.7240**	0.0659	0.1270*	0.2840*	0.0588	0.1710*	0.4228	0.2658	0.3100*	0.8410*	0.6900*	0.5212
3 Days	0.6620*	0.6620*	0.8697	0.5501	0.4825	0.8911	0.1110*	0.4130*	0.4411	0.3948	0.1293	0.8879	0.2570*	0.7411	0.5234	0.6027	0.7620*	0.7822
5 Days	0.2948	0.3100*	0.0559	0.0486	0.8180*	0.0204	0.1800*	0.1075	0.1955	0.6792	0.4850*	0.3571	0.8180*	0.1800*	0.5890**	0.3940*	0.3940*	0.3288
7 Days	0.8360*	0.4973	0.5340*	0.7619	0.3175	0.6786	0.6280*	0.8360**	0.5340*	0.3773	0.3660*	0.4787	0.9020*	0.5748	0.4560*	0.3830*	0.7100*	0.6706
14 Days	1.0000*	0.8360*	0.3360	0.2643	0.5289	0.4838	0.8180*	0.8180*	0.4850*	0.1086	0.8180*	0.2171	0.9450*	0.6280*	0.4116	0.4965	0.9450*	0.4447
28 Days	0.5360*	0.8670*	0.7790*	0.0028	1.0000*	0.0021	0.3830*	0.3180*	0.2590*	0.6534	1.0000*	0.3612	0.6940*	0.3970*	0.4926	0.0242	0.9550*	0.0307
42 Days	0.3660**	0.4450*	0.3738	0.6311	0.9450*	0.9394	0.5117	0.0989	0.5553	0.7917	0.8180*	0.6537	0.4338	0.2485	0.8001	0.4144	1.0000	0.5805

Table 3.12: T test analysis of comparing number densities of cell types present within tissue zones from 1 hour to 42 days post injury.

Injury Time		POST SURGICAL ADHESION						PERITONEAL TISSUE GENERATION						UTERINE HORN TISSUE GENERATION					
		Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.
1 Hour	Mean	N/A	N/A	N/A	N/A	N/A	N/A	1693287.71	627556.58	550570.14	0.00	97030.74	2968445.16	167590.62	47392.62	68115.72	395.62	33458.25	316952.84
	%	0.00	0.00	0.00	0.00	N/A	0.00	57.04	21.14	18.55	0.00	3.27	100.00	52.88	14.95	21.49	0.12	10.56	100.00
	STD	N/A	N/A	N/A	N/A	N/A	N/A	2760841.51	804457.22	1003822.39	0.00	205190.19	3387014.31	117932.56	80293.60	120694.59	734.54	83534.26	227572.48
	SEM	0.00	0.00	0.00	0.00	N/A	0.00	976104.88	284418.58	354904.81	0.00	72545.69	1197490.39	41695.46	28388.07	42671.98	259.70	29533.82	80459.02
	CV%	N/A	N/A	N/A	N/A	N/A	N/A	57.65	45.32	64.46	N/A	74.77	40.34	24.88	59.90	62.65	65.64	88.27	25.39
4 Hours	Mean	700239.05	2234500.31	274248.54	29372.71	43793.87	3282154.49	1104027.30	3018045.25	205674.49	16271.84	35872.83	4379891.71	353850.41	1055128.94	305744.94	0.00	29105.16	1743829.44
	%	21.33	68.08	8.36	0.89	1.33	100.00	25.21	68.91	4.70	0.37	0.82	100.00	20.29	60.51	17.53	0.00	1.67	100.00
	STD	792376.86	2478295.46	142370.14	37246.48	54845.18	3386881.43	732801.76	1322155.21	245915.82	20020.13	34230.57	1764466.64	324057.48	1041321.08	708192.79	0.00	47810.55	2009109.17
	SEM	457478.99	1430844.55	82197.44	21504.26	31664.88	1955416.90	259084.55	467452.46	86944.37	7078.18	12102.34	623833.16	122482.21	393582.37	267671.71	0.00	18070.69	759371.89
	CV%	65.33	64.03	29.97	73.21	72.30	59.58	23.47	15.49	42.27	43.50	33.74	14.24	34.61	37.30	87.55	#DIV/0!	62.09	43.55
8 Hours	Mean	685097.21	2900985.07	130031.21	2645.09	36725.07	3755483.66	933690.96	2407225.59	280537.69	0.00	51207.33	3672661.57	761388.24	3316596.54	148813.22	13279.02	48344.10	4288421.12
	%	18.24	77.25	3.46	0.07	0.98	100.00	25.42	65.54	7.64	0.00	1.39	100.00	17.75	77.34	3.47	0.31	1.13	100.00
	STD	499577.52	1236486.42	96070.34	4937.97	46410.29	1582495.03	887924.85	1260333.85	197585.56	0.00	113289.11	2227320.69	806976.49	2964756.92	262865.79	21650.39	86553.71	3657316.30
	SEM	176627.33	437163.97	33966.00	1745.84	16408.51	559496.49	397092.06	563638.43	88362.95	0.00	50664.43	996088.09	403488.24	1482378.46	131432.90	10825.20	43276.85	1828658.15
	CV%	25.78	15.07	26.12	66.00	44.68	14.90	42.53	23.41	31.50	N/A	98.94	27.12	52.99	44.70	88.32	81.52	89.52	42.64
16 Hours	Mean	3063127.88	10519691.99	824825.63	164797.76	118289.60	14690732.85	5647984.84	15182536.11	839487.18	0.00	0.00	21670008.13	667722.69	1694683.95	216806.02	74373.46	23738.76	2677324.88
	%	20.85	71.61	5.61	1.12	0.81	100.00	26.06	70.06	3.87	0.00	0.00	100.00	24.94	63.30	8.10	2.78	0.89	100.00
	STD	1094924.49	2924408.09	699249.22	212883.19	107975.97	3825285.40	3658003.95	8653529.11	53923.34	0.00	0.00	11783564.34	272868.98	113771.10	230536.80	29106.74	33571.68	93424.87
	SEM	387114.27	1033934.40	247221.93	75265.57	38175.27	1352442.64	2111949.57	4996117.36	31132.65	0.00	0.00	6803244.04	192947.50	80448.32	163014.13	20581.58	23738.76	66061.36
	CV%	12.64	9.83	29.97	45.67	32.27	9.21	37.39	32.91	3.71	N/A	#DIV/0!	31.39	28.90	4.75	75.19	27.67	100.00	2.47
1 Day	Mean	2498721.45	9439377.03	600711.49	80670.38	88473.21	12707953.56	1673319.85	7136917.04	283312.83	52607.02	15563.04	9161719.79	348051.29	792141.64	172718.17	26398.53	4399.76	1343709.39
	%	19.66	74.28	4.73	0.63	0.70	100.00	18.26	77.90	3.09	0.57	0.17	100.00	25.90	58.95	12.85	1.96	0.33	100.00
	STD	1269200.39	7359024.84	898214.64	140422.45	70507.62	9418937.21	945236.56	5072335.67	289605.93	76315.70	25081.66	5866211.67	407431.75	591713.83	370830.82	59028.92	9838.15	1026658.97
	SEM	448730.10	2601808.18	317566.83	49646.83	24928.21	3330097.19	422722.64	2268417.47	129515.71	34129.42	11216.86	2623449.61	182209.02	264622.47	165840.59	26398.53	4399.76	459135.85
	CV%	17.96	27.56	52.87	61.54	28.18	26.20	25.26	31.78	45.71	64.88	72.07	28.63	52.35	33.41	96.02	100.00	100.00	34.17
3 Days	Mean	2784981.99	1948364.23	6485647.27	6043541.37	418652.76	17681187.62	2887763.60	1522400.01	7134924.57	8933057.60	313165.36	20791311.14	63176.36	186581.91	440662.95	1001007.95	4432.76	1695861.93
	%	15.75	11.02	36.68	34.18	2.37	100.00	13.89	7.32	34.32	42.97	1.51	100.00	3.73	11.00	25.98	59.03	0.26	100.00
	STD	3835444.94	3095995.24	9664338.58	9903252.99	462146.33	23340435.41	4073160.89	1656747.33	5300516.87	5362673.69	403421.42	10029492.61	59127.20	364712.16	668427.69	1029420.33	5150.17	1722217.24
	SEM	1715263.12	1384571.16	4322023.61	4428869.38	206678.12	10438160.04	1662860.97	676364.27	2163926.95	2189302.37	164696.10	4094523.21	29563.60	182356.08	334213.84	514710.17	2575.08	861108.62
	CV%	61.59	71.06	66.64	73.28	49.37	59.04	57.58	44.43	30.33	24.51	52.59	19.69	46.80	97.74	75.84	51.42	58.09	50.78
5 Days	Mean	2809130.22	2722696.63	11519196.55	31399188.23	214414.83	48664626.46	3500094.43	814381.92	12915895.50	42585121.72	79450.16	59894943.73	1674062.08	199986.50	6454443.91	24380197.30	103287.96	32811977.75
	%	5.77	5.59	23.67	64.52	0.44	100.00	5.84	1.36	21.56	71.10	0.13	100.00	5.10	0.61	19.67	74.30	0.31	100.00
	STD	2738849.45	4433663.37	10135849.80	26508737.17	517821.25	42067573.90	4022830.31	730951.32	9576164.46	30897601.07	130775.65	39416209.86	2607257.08	278505.14	4848272.84	17849487.53	111210.84	24342945.43
	SEM	1118130.60	1810035.49	4137943.35	10822146.63	211399.64	17174015.13	1642313.60	298409.63	3909452.77	12613892.82	53388.93	16091600.29	1064408.24	113699.25	1979299.10	7287022.77	45401.64	9937965.86
	CV%	39.80	66.48	35.92	34.47	98.59	35.29	46.92	36.64	30.27	29.62	67.20	26.87	63.58	56.85	30.67	29.89	43.96	30.29
7 Days	Mean	1280034.85	375756.88	5243468.78	17515439.79	42894.94	24457595.25	2333113.12	1167865.85	14003229.81	35823816.48	232645.53	53560670.79	1277550.56	171638.70	2390967.20	10463677.17	120992.63	14424826.26
	%	5.23	1.54	21.44	71.62	0.18	100.00	4.36	2.18	26.14	66.88	0.43	100.00	8.86	1.19	16.58	72.54	0.84	100.00
	STD	3010014.61	267494.19	6354389.50	12730216.57	66767.26	21606162.63	5772936.49	1683684.35	16246549.54	19079487.10	282025.97	34471147.71	3275764.57	211529.35	3408435.11	4173878.73	180897.84	6262467.05
	SEM	1228833.32	109204.05	2594168.65	5197089.15	27257.62	8820678.96	2181964.90	636372.87	6140618.53	7211368.29	106595.80	13028869.18	1238122.63	79950.58	1288267.38	1577577.88	68372.96	2366990.06
	CV%	96.00	29.06	49.47	29.67	63.55	36.07	93.52	54.49	43.85	20.13	45.82	24.33	96.91	46.58	53.88	15.08	56.51	16.41
14 Days	Mean	359612.23	85574.82	1883071.39	14146004.12	104667.03	16578929.59	5243388.35	343890.51	4373616.30	77546268.06	231047.87	87738211.09	102061.08	9556.78	237405.32	2584489.71	42277.52	2974590.66
	%	2.17	0.52	11.36	85.33	0.63	100.00	5.98	0.39	4.98	88.38	0.26	100.00	3.43	0.32	7.98	86.89	1.42	100.04
	STD	750183.07	100520.16	1676734.35	10606665.18	126475.38	11601712.98	13645999.81	449391.45	5857377.55	78718999.16	458712.60	91979728.11	247688.75	22271.28	255398.64	1707790.80	88061.93	2124145.35
	SEM	306260.96	41037.18	684523.93	4330152.93	51633.36	4736379.49	5157703.13	169854.00	2213880.62	29752985.03	173377.07	34765069.46	101118.51	9092.21	104266.06	697202.68	35951.13	867178.71
	CV%	85.16	47.95	36.35	30.61	49.33	28.57	98.37	49.39	50.62	38.37	75.04	39.62	99.08	95.14	43.92	26.98	85.04	29.15
28 Days	Mean	391653.05	46528.35	805942.74	10039512.65	29302.18	11312938.97	4580.66	40263.08	2350059.71	24380540.38	147274.85	26922718.69	0.00	6244.04	213699.37	5851639.05	63138.63	6134721.09
	%	3.46	0.41	7.12	88.74	0.26	100.00	0.02	0.15	8.73	90.56	0.55	100.00	0.00	0.10	3.48	95.39	1.03	100.00
	STD	1001642.44	74405.59	1180157.21	7203316.84	38135.18	7822459.03	12956.07	1511.32	9088.63	48134.72	4498.52	48543.89	0.00	347.79	8208.02	96058.23	2013.44	95267.67
	SEM	378585.26	28122.67	446057.50	2722597.85	14413.74	2956611.61	4580.66	534.33	3213.32	17018.19	1590.47	17162.86	0.00	131.45	3102.34	36306.60	761.01	36007.79
	CV%	96.66	60.44	55.35	27.12	49.19	26.13	100.00	1.33	0.14	0.07	1.08	0.06	#DIV/0!	2.11	1.45	0.62	1.21	0.59
42 Days	Mean	685077.98	48406.10	646728.43	13215070.46	43529.62	14638812.58	1454686.84	136416.21	1094686.46	11493278.48	142628.52	14321696.52	568464.95	151430.89	574958.14	7898778.19	46234.98	9239867.16
	%	4.68	0.33	4.42	90.27	0.30	100.00	10.16	0.95	7.64	80.25	1.00	100.00	6.15	1.64	6.22	85.49	0.50	100.00
	STD	1282904.06	118570.23	1199969.81	17139539.86	71154.69	18913763.65	1536070.48	225496.00	487632.24	5733094.53	314570.02	6544820.75	631220.44	186948.31	367112.79	4620107.84	84922.23	5542614.02
	SEM	523743.39	48406.10	489885.62	6997187.85	29048.78	7721511.68	580580.07	85229.48	184307.66	2166906.05	118896.29	2473709.73	257694.66	76321.33	149873.17	1886151.13	34669.35	2262762.70
	CV%	76.45	100.00	75.75	52.95	66.73	52.75	39.91	62.48	16.84	18.85	83.36	17.27	45.33	50.40	26.07	23.88	74.99	24.49

Table 3.13: Mean number totals of cell types within PSAs, PTG and UTG from 1hour to 42 days, together with mean percentages, SDs, SEMs and CV%.

Injury Time	P Value Post Surgical Adhesion						P Value Peritoneal Tissue Generation						P Value Uterine Horn Tissue Generation					
	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.
1-4 Hours	-	-	-	-	-	-	0.8670*	0.0015	0.7790*	0.1210*	0.5387	0.0289*	0.5350**	0.0006*	0.7100*	0.3830*	0.9020*	0.0070*
4-8 Hours	0.9700	0.5531	0.0802	0.1940*	0.8340	0.9210**	0.8694	0.4350*	0.0653*	0.1710*	0.7240*	0.6220*	0.2137	0.1640**	1.0000*	0.2300*	1.0000*	0.1221
8-16 Hours	<0.0001	<0.0001	0.0104*	0.0207*	0.1610*	0.0002**	0.0316	0.1430*	0.0008	1.0000	0.3930*	0.0392	0.8713	0.8000**	0.5330*	0.0411	0.7302	1.0000**
16 Hours-1 Day	0.3571	0.7054	0.3280*	0.5050*	0.5237	0.5899	0.0518	0.1405	0.0357*	0.2916	0.3382	0.0852	0.3810*	0.0982	0.3810*	0.3810*	0.5710*	0.1434
1-3 Days	0.5749	0.0295*	0.0062*	0.0031*	0.0932**	0.8330*	0.9310*	0.0519**	0.0043*	0.0043*	0.1770*	0.0488	0.0635*	0.1110*	0.1110*	0.0685	0.7300*	0.7127
3-5 Days	0.7999	1.0000*	0.5941	0.0876	0.0823*	0.2294	0.8180*	0.3608	0.2248	0.0260**	0.3940*	0.2400**	0.0667*	0.9140*	0.0190**	0.0095**	0.6100**	0.0095**
5-7 Days	0.1320*	0.3940*	0.2270	0.2734	0.8180*	0.2374	0.2950*	0.6441	0.8885	0.6384	0.2489	0.7628	0.1810*	0.8490	0.0916	0.0734**	0.8360*	0.1010**
7-14 Days	0.9370*	0.0321	0.2400*	0.6292	0.3940*	0.4496	0.7100*	0.2348	0.0728*	0.1280*	0.7100*	0.5350*	0.4450*	0.0904	0.0047*	0.0012**	0.3660*	0.0012**
14-28 Days	0.5340*	0.6280*	0.4450*	0.4252	0.1596	0.3513	0.2810*	0.1890*	0.0939*	0.0140*	0.8670*	0.0093*	0.3660*	0.6280*	0.7310*	0.3900	0.8360*	0.4311
28-42 Days	0.4450*	0.6280*	1.0000*	0.3704	0.9450*	0.3616	0.0939*	0.6130*	0.2320*	0.5360*	0.8670*	0.2810*	0.1380*	0.2340*	0.0888	0.1380*	0.9450*	0.1380*

Table 3.14: T test analysis comparing number totals of cell types with PSAs, PTG and UTG between concurrent time points.

Injury Time	P Value PSA vs PTG						P Value PSA vs UTG						P Value PTG vs UTG					
	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.
1 Hour	-	-	-	-	-	-	-	-	-	-	-	-	0.0175*	0.0012*	0.0023*	0.3830*	0.5350*	0.0006*
4 Hours	0.4448	0.5009	0.4970*	0.4570	0.7752	0.4855	0.3314	0.2972	0.1830*	0.1170*	0.5170*	0.3854	0.0269	0.0075	0.1210*	0.1210*	0.5360*	0.0140*
8 Hours	0.1786	0.6220*	0.0074	0.5240*	0.8330*	0.1555	0.8312	0.9330**	0.2830*	0.5700*	0.6830*	0.8080**	0.4235	0.7300*	0.1970	0.2860*	1.0000*	0.4479
16 Hours	0.4970**	0.3760**	0.9728	0.0848*	0.0848*	0.4970**	0.0186	0.0035	0.2769	0.5820	0.2734	0.0028	0.1656	0.1276	0.2000*	0.0167	0.2722	0.1193
1 Day	0.2386	0.5542	0.4350*	0.6220*	0.0505	0.4696	0.0040	0.0016*	0.0653*	0.2220*	0.0244	0.0016*	0.0205	0.0159**	0.1510*	0.6900*	0.6900*	0.0159**
3 Days	0.7920*	0.7920*	0.8207	0.4290*	0.4290*	0.9545	0.0159*	0.0635*	0.0159*	0.0635*	0.1037	0.0317*	0.0667*	0.1580	0.0095**	0.0209	0.2570*	0.0095**
5 Days	0.7369	0.8180*	0.8109	0.5157	0.8180*	0.6430	0.5092	0.0931*	0.2888	0.5925	0.4850*	0.4336	0.3940*	0.0874	0.1658	0.2310	0.8180*	0.1746
7 Days	0.5340*	0.3660*	0.2423	0.0715	0.1378	0.0350*	0.9450*	0.1524	0.2950*	0.1920	0.5340*	0.2629	0.5350*	0.0728*	0.0175*	0.0023*	0.3953	0.0012*
14 Days	0.9450*	0.1978	0.4450*	0.0047*	0.7310*	0.0023*	0.5890*	0.1006	0.1320*	0.0931**	0.3448	0.0649**	0.7310*	0.2340*	0.0221*	0.0012*	0.6280*	0.0012*
28 Days	0.5360*	0.9550*	0.7790*	1.0000*	0.9550*	1.0000*	0.3830*	0.3830*	0.1650*	0.3475	0.9020*	0.2790	0.6940*	0.4630*	0.0401*	0.0939*	0.8670*	0.0721*
42 Days	0.5111	0.4450*	0.9072	0.4731	1.0000*	0.5879	0.5560	0.3100*	0.4322	0.2445	1.0000*	0.6990**	0.4450**	0.9450*	0.0558	0.2447	1.0000*	0.1630

Table 3.15: T test analysis of comparing number totals of cell types present within tissue zones from 1 hour to 42 days post injury.

Cell Type	Morphology	Diameter (μm)	Mean Number (x 10^9/l)	95% Lower (x 10^9/l)	95% Upper (x 10^9/l)	% of WBC Total
Erythrocytes	Anuclear, stained pink.	6.7-6.9	5.35 x 10^3/l	4.23 x 10^3/l	6.47 x 10^3/l	-
Platelets	A group of reddish-violet granules surrounded by pale blue cytoplasm.	1-3	342	151	533	-
White Blood Cells			7.7*	4.4	13.4	100
Neutrophils	Nucleus is segmented stains a deep purple-blue colour; segments are connected by fine strands of chromatin. Cytoplasm is almost colourless and contains two types of granules: the smallest granules stain a pink colour, giving the cytoplasm an overall pinkish colour, the second types stain a deep pinkish-red colour and are quite large.	10-15	1.9*	0.6	5.8	25
Eosinophils	Nucleus stains a deep-purple colour and maybe bilobed or horseshoe shaped. Cytoplasm is completely filled with large granules which have a foamy appear and stain a dull pinky-orange colour	12-16	0	0	0.2	0
Lymphocytes	Nucleus is round or oval which may have a slight indention on one side and stains a deep purple-blue colour. Cytoplasm, stains medium to deep blue, of the small lymphocyte forms only a narrow rim around the nucleus and may appear to be almost non-existent, whereas large lymphocytes have more abundant cytoplasm.	Small: 7-10 Large: 10-15	4.9*	2.6	9.2	64
Monocytes	Nucleus may be lobulated, horseshoe-shaped or bean-shaped and stains a purple-blue colour with a lace-like or stringy appearance. Cytoplasm is plentiful and stains a grey-blue colour with a mottled appearance.	15-18	0.4	0	0.9	5
Basophils	Nucleus is usually obscured by the granules in the cytoplasm but when visible stains a purple-blue colour and may be a band form or segmented structure. Cytoplasm stains a pale grey-blue colour and is filled with large purple-black granules which overlay the nucleus.	10-15	0.3	0.0	0.7	4

Table 3.16: Rabbit blood cell details
(Adapted from Sanderson & Phillips, 1981). * = log transform

	$x10^9/l$			
Cell Type	Mean	SD	SEM	CV%
Blood Sample 1				
RBCs ($x10^3/l$)	5.31	0.65	0.08	1.51
Platelets	323.92	144.82	18.39	5.68
WBCs	6.62	2.59	0.33	4.98
Basophilic Cells	0.11	0.11	0.03	23.62
Lymphocytes	3.28	1.40	0.34	10.34
Monocytes	0.42	0.21	0.05	11.82
Eosinophilic Cells	2.18	0.75	0.18	8.37
Blood Sample 2				
RBCs ($x10^3/l$)	5.27	0.78	0.10	1.90
Platelets	272.52	143.73	18.56	6.81
WBCs	6.00	2.30	0.30	5.00
Basophilic Cells	0.02	0.03	0.01	40.57
Lymphocytes	3.13	1.45	0.35	11.24
Monocytes	0.51	0.46	0.11	21.82
Eosinophilic Cells	2.63	1.56	0.38	14.44
Blood Sample 3				
RBCs ($x10^3/l$)	4.99	0.61	0.08	1.60
Platelets	311.31	158.53	20.64	6.63
WBCs	5.96	3.23	0.42	7.06
Basophilic Cells	0.08	0.15	0.02	24.22
Lymphocytes	2.39	1.35	1.18	7.35
Monocytes	0.30	0.28	0.04	11.86
Eosinophilic Cells	3.19	2.52	0.33	10.31

Table 3.17: Mean numbers for full and differential blood cell counts for blood samples 1, 2 and 3 together with STD, SEM and CV%.

	Basophilic Cells	Lymphocytes	Macrophages	Eosinophilic Cells	WBCs	RBCs	Platelets
Blood sample 1 vs 2	0.0083*	0.7652	0.7570*	0.2901	0.2689	0.6630*	0.1310*
Blood sample 1 vs 3	0.1010*	0.0164*	0.0159*	0.3470*	0.0975*	0.0064*	0.0137*
Blood sample 2 vs 3	0.0658*	0.0415*	0.0292*	0.7743*	0.4360*	0.0051*	0.1683

Table 3.18: T test analysis of full and differential blood cell counts for blood samples 1, 2 and 3.
* = normality test failed; Mann Whitney rank sum test ran.

Injury Time		Number of White Blood Cell ($x10^9$/l)					RBCs ($x10^{12}$/l)	Platelets ($x10^9$/l)
		Basophilic Cells	Lymphocytes	Monocytes	Eosinophilic Cells	Total		
30 Secs	**MEAN**	**0.02**	**1.99**	**0.27**	**1.42**	**3.70**	**4.59**	**193.50**
	STD	0.02	0.50	0.05	0.71	0.78	0.46	114.57
	SEM	0.01	0.25	0.03	0.36	0.39	0.23	57.29
	CV%	60.89	12.53	9.65	25.03	10.58	5.02	29.61
1 Min	**MEAN**	**0.04**	**1.99**	**0.19**	**1.39**	**3.60**	**4.37**	**389.67**
	STD	0.03	0.26	0.10	0.99	0.95	0.44	141.09
	SEM	0.02	0.15	0.06	0.57	0.55	0.25	81.46
	CV%	51.10	7.50	29.80	41.05	15.30	5.82	20.90
3 Mins	**MEAN**	**0.03**	**2.52**	**0.24**	**1.70**	**4.50**	**5.27**	**258.25**
	STD	0.04	1.96	0.19	0.69	2.72	0.20	213.44
	SEM	0.02	0.98	0.09	0.34	1.36	0.10	106.72
	CV%	65.20	38.75	38.98	20.21	30.20	1.85	41.33
5 Mins	**MEAN**	**0.02**	**1.35**	**0.14**	**2.02**	**3.53**	**4.92**	**348.25**
	STD	0.03	0.94	0.09	1.60	2.56	0.65	139.75
	SEM	0.01	0.47	0.04	0.80	1.28	0.33	69.88
	CV%	76.54	34.88	31.50	39.65	36.34	6.64	20.07
10 Mins	**MEAN**	**0.04**	**1.78**	**0.23**	**1.75**	**3.80**	**4.54**	**155.50**
	STD	0.01	0.09	0.23	0.38	0.71	0.21	171.83
	SEM	0.00	0.07	0.16	0.27	0.50	0.15	121.50
	CV%	13.16	3.73	70.86	15.29	13.16	3.30	78.14
30 Mins	**MEAN**	**0.03**	**0.61**	**0.12**	**2.91**	**3.68**	**4.83**	**397.00**
	STD	0.02	0.23	0.09	1.53	1.73	0.53	73.01
	SEM	0.01	0.12	0.04	0.76	0.87	0.26	36.51
	CV%	37.71	19.12	36.64	26.18	23.60	5.46	9.20
1 Hour	**MEAN**	**0.01**	**1.10**	**0.15**	**1.47**	**2.73**	**4.86**	**341.67**
	STD	0.02	0.47	0.07	0.76	1.29	0.15	227.35
	SEM	0.01	0.27	0.04	0.44	0.74	0.09	131.26
	CV%	100.00	24.65	25.29	29.88	27.24	1.79	38.42
4 Hours	**MEAN**	**0.00**	**1.88**	**0.13**	**3.84**	**5.85**	**5.56**	**239.50**
	STD	0.00	0.78	0.04	2.07	2.14	0.47	212.86
	SEM	0.00	0.39	0.02	1.03	1.07	0.23	106.43
	CV%	#DIV/0!	20.77	17.13	26.92	18.31	4.22	44.44
8 Hours	**MEAN**	**0.00**	**2.23**	**0.14**	**9.13**	**11.50**	**5.08**	**498.00**
	STD	0.00	0.81	0.17	4.22	5.16	0.52	78.60
	SEM	0.00	0.40	0.08	2.11	2.58	0.26	39.30
	CV%	#DIV/0!	18.07	57.91	23.10	22.45	5.12	7.89
16 Hours	**MEAN**	**0.08**	**3.09**	**0.28**	**6.48**	**9.93**	**5.13**	**347.67**
	STD	0.14	0.15	0.36	2.23	1.95	0.38	97.68
	SEM	0.08	0.09	0.21	1.29	1.13	0.22	56.40
	CV%	100.00	2.86	73.68	19.89	11.34	4.26	16.22
1 Day	**MEAN**	**0.00**	**4.34**	**0.19**	**3.32**	**7.85**	**4.73**	**354.75**
	STD	0.00	0.77	0.13	1.12	1.56	0.12	75.35
	SEM	0.00	0.39	0.07	0.56	0.78	0.06	37.68
	CV%	#DIV/0!	8.90	35.42	16.82	9.94	1.22	10.62
3 Days	**MEAN**	**0.06**	**4.44**	**0.25**	**1.89**	**6.63**	**4.36**	**268.00**
	STD	0.05	2.31	0.23	0.59	2.65	0.78	256.50
	SEM	0.03	1.33	0.13	0.34	1.53	0.45	148.09
	CV%	50.51	30.00	51.91	17.97	23.10	10.26	55.26
5 Days	**MEAN**	**0.26**	**2.36**	**0.62**	**4.20**	**7.40**	**5.11**	**282.75**
	STD	0.32	1.45	0.17	2.01	3.62	0.68	207.65
	SEM	0.23	1.03	0.12	1.42	2.56	0.48	146.83
	CV%	88.54	43.40	19.76	33.82	34.60	9.47	51.93
7 Days	**MEAN**	**0.17**	**3.05**	**0.60**	**3.35**	**7.40**	**4.94**	**362.00**
	STD	0.06	1.28	0.48	1.34	2.34	0.45	126.40
	SEM	0.03	0.64	0.24	0.67	1.17	0.23	63.20
	CV%	19.19	20.96	40.23	19.98	15.83	4.59	17.46
14 Days	**MEAN**	**0.34**	**2.58**	**0.49**	**3.43**	**6.83**	**5.72**	**281.00**
	STD	0.29	0.99	0.14	1.58	2.31	0.91	130.22
	SEM	0.17	0.57	0.08	0.91	1.33	0.52	75.18
	CV%	48.50	22.29	16.35	26.56	19.53	9.17	26.75
28 Days	**MEAN**	**0.20**	**2.99**	**0.62**	**1.94**	**5.70**	**5.14**	**264.50**
	STD	0.27	1.03	0.54	1.39	2.94	1.02	189.49
	SEM	0.14	0.52	0.27	0.70	1.47	0.51	94.74
	CV%	66.67	17.32	43.30	35.87	25.75	9.89	35.82
42 Days	**MEAN**	**0.11**	**2.31**	**0.58**	**2.31**	**6.60**	**5.91**	**204.33**
	STD	0.05	1.29	0.02	1.19	2.92	0.68	90.01
	SEM	0.04	0.91	0.02	0.84	2.07	0.48	63.64
	CV%	33.33	39.39	3.03	36.36	31.29	8.09	31.15

Table 3.19: Mean numbers for full and differential blood cell counts for blood sample 3 at time points following injury, together with SD, SEM and CV%.

Sample Time	Blood Sample 1	Blood Sample 2	Control Blood Sample 3	Peritoneal Sample 1	Control Peritoneal Sample 2
1 Hour	-	-	3617.14	-	-
4 Hours	-	-	2845.71	-	-
8 Hours	-	-	6411.43	-	-
16 Hours	-	-	2794.29	-	-
1 Day	-	-	4191.43	-	-
3 Days	-	-	12257.14	-	-
5 Days	-	-	2211.43	-	3171.43
7 Days	-	-	6265.71	-	6565.71
14 Days	-	-	2400.00	-	3171.43
28 Days	-	-	4834.29	-	1705.71
42 Days	-	-	3522.86	-	-
Mean	**4568.57**	**2997.14**	-	**1740.00**	-
STD	2877.51	696.05	-	193.95	-
SEM	1661.33	401.86	-	137.14	-
CV%	36.36	13.41		7.88	

Table 3.20: TGF-β concentrations within plasma and peritoneal fluid samples.

APPENDIX IV

Injury Time		Adhesion	Tissue Generation				Damage Zone		Peritoneal Reaction Zone	Peritoneal Cell Conc	Uterine Horn Haemorrhage	Fibrin Sealant Remaining	F/Sealant Associated Cell Conc.
			Peritoneal	Uterine Horn	Free Floating	Total	Peritoneal	Uterine Horn					
1 HOUR	MEAN	0.00	4.50	0.37	0.00	4.88	17.26	4.14	9.15	0.00	2.27	79.04	0.00
	STD	0.00	1.89	0.38	0.00	1.92	2.52	0.99	1.98	0.00	1.80	12.87	0.00
	SEM	0.00	0.67	0.13	0.00	0.68	0.89	0.35	0.70	0.00	0.64	4.55	0.00
	%CV	N/A	14.87	35.69	N/A	13.93	5.17	8.45	7.67	N/A	28.04	5.76	N/A
4 HOURS	MEAN	0.00	7.88	1.20	0.00	9.08	17.11	6.23	19.96	0.10	0.99	50.66	0.00
	STD	0.00	2.70	1.46	0.00	2.90	7.90	3.99	5.28	0.28	0.84	11.37	0.00
	SEM	0.00	0.96	0.51	0.00	1.02	2.79	1.41	1.87	0.10	0.30	4.02	0.00
	%CV	N/A	12.14	42.86	N/A	11.29	16.32	22.63	9.35	100.00	29.98	7.93	N/A
8 HOURS	MEAN	0.00	15.29	3.30	0.00	18.59	14.31	7.85	32.77	0.00	0.65	41.64	0.10
	STD	0.00	8.91	2.15	0.00	8.26	3.75	2.99	7.54	0.00	0.49	14.62	0.28
	SEM	0.00	3.15	0.76	0.00	2.92	1.33	1.06	2.67	0.00	0.17	5.17	0.10
	%CV	N/A	20.59	23.04	N/A	15.72	9.28	13.47	8.14	N/A	26.61	12.41	100.00
16 HOURS	MEAN	0.00	12.14	5.99	0.00	18.13	11.29	7.63	28.82	0.24	1.64	32.20	0.21
	STD	0.00	4.83	1.85	0.00	6.17	3.91	2.86	9.06	0.69	2.90	8.23	0.55
	SEM	0.00	1.71	0.65	0.00	2.18	1.38	1.01	3.20	0.24	1.02	2.91	0.19
	%CV	N/A	14.08	10.89	N/A	12.03	12.25	13.26	11.12	100.00	62.51	9.03	93.16
1 DAY	MEAN	0.00	29.55	13.00	0.00	42.55	21.16	14.56	58.41	0.00	0.36	45.73	0.29
	STD	0.00	9.24	6.31	0.00	12.40	10.63	7.73	23.97	0.00	0.48	16.23	0.58
	SEM	0.00	3.27	2.23	0.00	4.39	3.76	2.73	8.48	0.00	0.17	5.74	0.21
	%CV	N/A	11.06	17.16	N/A	10.31	17.76	18.76	14.51	N/A	46.44	12.54	70.15
3 DAYS	MEAN	0.00	28.67	3.13	0.00	31.80	34.74	2.14	23.11	0.09	0.00	43.44	37.12
	STD	0.00	6.77	1.26	0.00	7.67	15.82	1.23	7.30	0.27	0.00	9.99	17.06
	SEM	0.00	2.39	0.44	0.00	2.71	5.59	0.44	2.58	0.09	0.00	3.53	6.03
	%CV	N/A	8.35	14.20	N/A	8.53	16.11	20.36	11.17	100.00	N/A	8.13	16.25
5 DAYS	MEAN	0.00	71.32	5.52	0.14	76.99	44.03	1.21	46.59	3.71	0.07	42.35	47.20
	STD	0.00	30.41	2.65	0.39	30.32	19.33	1.06	19.06	5.09	0.12	16.34	34.91
	SEM	0.00	10.75	0.94	0.14	10.72	6.83	0.38	6.74	1.80	0.04	5.78	12.34
	%CV	N/A	15.08	16.97	100.00	13.93	15.52	31.10	14.47	48.40	65.47	13.64	26.16
7 DAYS	MEAN	0.00	76.08	7.12	1.30	84.50	32.33	0.81	45.59	1.70	0.00	18.77	34.88
	STD	0.00	30.17	3.23	1.92	32.54	6.64	0.88	9.34	1.40	0.00	9.86	8.28
	SEM	0.00	10.67	1.14	0.68	11.50	2.35	0.31	3.30	0.49	0.00	3.49	2.93
	%CV	N/A	14.02	16.02	52.16	13.61	7.26	38.65	7.24	29.09	N/A	18.58	8.40
14 DAYS	MEAN	0.00	57.63	3.14	0.55	61.32	14.78	0.57	18.24	0.02	0.00	0.92	3.73
	STD	0.00	17.37	3.91	0.77	19.55	6.48	0.99	6.59	0.07	0.00	0.91	2.98
	SEM	0.00	6.14	1.38	0.27	6.91	2.29	0.35	2.33	0.02	0.00	0.32	1.05
	%CV	N/A	10.65	44.03	49.22	11.27	15.51	60.86	12.77	100.00	N/A	35.03	28.27
28 DAYS	MEAN	4.71	43.65	5.24	0.00	53.61	10.19	0.45	20.56	0.00	0.00	2.97	5.44
	STD	11.83	16.64	3.65	0.00	11.01	2.91	0.93	9.14	0.00	0.00	5.46	8.02
	SEM	4.18	5.88	1.29	0.00	3.89	1.03	0.33	3.23	0.00	0.00	1.93	2.84
	%CV	88.75	13.47	24.65	N/A	7.26	10.11	72.32	15.72	N/A	N/A	64.96	52.15
42 DAYS	MEAN	0.00	37.45	4.16	0.71	42.32	6.46	0.42	15.05	0.00	0.00	0.00	0.00
	STD	0.00	16.56	3.52	1.75	17.83	4.56	0.78	7.78	0.00	0.00	0.00	0.00
	SEM	0.00	5.86	1.24	0.62	6.30	1.61	0.27	2.75	0.00	0.00	0.00	0.00
	%CV	N/A	15.64	29.92	86.81	14.90	24.98	65.53	18.29	N/A	N/A	N/A	N/A

Table 4.1: Volumes of tissue zones, together with standard derivation, standard error of the mean and percentage coefficient of variation.

Injury Time	P VALUE										
	PSA	Tissue Generation			Damage Zone		Peritoneal Reaction Zone	Peritoneal Cell Conc	Uterine Horn Haemorrhage	Fibrin Sealant Remaining	F/Sealant Associated Cell Conc.
		Peritoneal	Uterine Horn	Total	Peritoneal	Uterine Horn					
1 Hr - 4 Hrs	1.0000	0.0118	0.1418	0.0049	0.8780**	0.5050**	<0.0001	0.7210*	0.0913	0.0004	1.0000
4 Hrs - 8 Hrs	1.0000	0.0650*	0.0383	0.0080	0.5740**	0.3730	0.0015	0.7210*	0.3311	0.1897	0.7210*
8 Hrs - 16 Hrs	1.0000	0.3946	0.0177	0.9024	0.1377	0.8818	0.3594	0.7210*	0.7980*	0.1339	0.7210*
16 Hrs - 1 Day	1.0000	0.0003	0.0379**	0.0002	0.0281**	0.0321	0.0056	0.7210*	0.8780*	0.0540	0.9590*
1 Day - 3 Days	1.0000	0.8310	0.0019*	0.0560	0.0636	0.0006**	0.0014	0.7210*	0.1050*	0.7385	0.0002*
3 Days - 5 Days	1.0000	0.0104**	0.0369	0.0011	0.2340*	0.1288	0.0070**	0.0650*	0.4420*	0.8745	0.4754
5 Days - 7 Days	1.0000	0.7581	0.1610*	0.6400	0.1050**	0.4243	0.8968	0.2981	0.4420*	0.0036	0.7210*
7 Days - 14 Days	1.0000	0.1560	0.0104*	0.1060	0.0001	0.1300*	<0.0001	0.0019*	1.0000	0.0002**	0.0002**
14 Days - 28 Days	0.4420*	0.1225	0.1950*	0.3476	0.0891	0.2340*	0.5696	0.7210*	1.0000	0.8780*	0.5802
28 Days - 42 Days	0.4420*	0.4668	0.5563	0.1498	0.0718	1.0000*	0.2152	1.0000	1.0000	0.1050*	0.1050*

Table 4.2: T test analysis between concurrent time points. * = Normality Test Failed; Mann-Whitney Rank Sum Test Ran; ** = Equal Variance Test Failed; Mann-Whitney Rank Sum Test Ran.

1 Day	MEAN	0.00	0.00	3.40	0.28	2.80	0.00	0.00	0.00	0.00	0.00
	STD	0.00	0.00	7.40	0.28	2.80	0.00	0.00	0.00	0.00	0.00
	SEM	0.00	0.00	2.66	0.10	0.99	0.00	0.00	0.00	0.00	0.00
	%CV	N/A	N/A	217.71	100.00	100.00	N/A	N/A	N/A	N/A	0.00
3 Days	MEAN	0.00	0.00	1.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	STD	0.00	0.00	2.93	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	SEM	0.00	0.00	0.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	%CV	N/A	N/A	277.43	0.00	0.00	N/A	N/A	N/A	N/A	0.00
5 Days	MEAN	0.00	0.00	1.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	STD	0.00	0.00	2.93	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	SEM	0.00	0.00	0.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	%CV	N/A	N/A	277.43	0.00	0.00	N/A	N/A	N/A	N/A	0.00
7 Days	MEAN	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	STD	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	SEM	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	%CV	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
14 Days	MEAN	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	STD	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	SEM	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	%CV	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
28 Days	MEAN	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	STD	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	SEM	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	%CV	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
42 Days	MEAN	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	STD	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	SEM	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	%CV	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 4.3: Mean volumes and percentages of %CV

Injury Time		PSA		Peritoneal Tissue Generation		Uterine Horn Tissue Generation		Free Floating Tissue Generation		Total Tissue Generation		Percentage	
		Fibrin	Collagen	Fibrin	Collagen	Fibrin	Collagen	Fibrin	Collagen	Fibrin	Collagen	Fibrin	Collagen
1 Hour	**MEAN**	**0.00**	**0.00**	**1.32**	**0.00**	**0.59**	**0.00**	**0.00**	**0.00**	**1.91**	**0.00**	**100.00**	**0.00**
	STD	0.00	0.00	1.27	0.00	0.49	0.00	0.00	0.00	1.37	0.00	0.00	0.00
	SEM	0.00	0.00	0.45	0.00	0.17	0.00	0.00	0.00	0.48	0.00	0.00	0.00
	%CV	N/A	N/A	33.89	N/A	29.35	N/A	N/A	N/A	25.39	N/A	0.00	N/A
4 Hours	**MEAN**	**0.00**	**0.00**	**1.39**	**0.00**	**0.73**	**0.00**	**0.00**	**0.00**	**2.12**	**0.00**	**100.00**	**0.00**
	STD	0.00	0.00	0.65	0.00	0.73	0.00	0.00	0.00	0.89	0.00	0.00	0.00
	SEM	0.00	0.00	0.23	0.00	0.26	0.00	0.00	0.00	0.31	0.00	0.00	0.00
	%CV	N/A	N/A	16.60	N/A	35.38	N/A	N/A	N/A	14.83	N/A	0.00	N/A
8 Hours	**MEAN**	**0.00**	**0.00**	**2.06**	**0.00**	**0.95**	**0.00**	**0.00**	**0.00**	**3.01**	**0.00**	**100.00**	**0.00**
	STD	0.00	0.00	0.72	0.00	1.03	0.00	0.00	0.00	1.50	0.00	0.00	0.00
	SEM	0.00	0.00	0.25	0.00	0.36	0.00	0.00	0.00	0.53	0.00	0.00	0.00
	%CV	N/A	N/A	12.28	N/A	38.30	N/A	N/A	N/A	17.70	N/A	0.00	N/A
16 Hours	**MEAN**	**0.00**	**0.00**	**3.02**	**0.00**	**1.41**	**0.00**	**0.00**	**0.00**	**4.43**	**0.00**	**100.00**	**0.00**
	STD	0.00	0.00	4.11	0.00	0.96	0.00	0.00	0.00	4.99	0.00	0.00	0.00
	SEM	0.00	0.00	1.45	0.00	0.34	0.00	0.00	0.00	1.76	0.00	0.00	0.00
	%CV	N/A	N/A	48.11	N/A	24.10	N/A	N/A	N/A	39.76	N/A	0.00	N/A
1 Day	**MEAN**	**0.00**	**0.00**	**9.59**	**0.25**	**4.78**	**0.00**	**0.00**	**0.00**	**14.37**	**0.25**	**98.54**	**1.46**
	STD	0.00	0.00	7.47	0.28	2.60	0.00	0.00	0.00	5.89	0.28	1.15	1.15
	SEM	0.00	0.00	2.64	0.10	0.92	0.00	0.00	0.00	2.08	0.10	0.41	0.41
	%CV	N/A	N/A	27.56	40.09	19.21	N/A	N/A	N/A	14.48	40.09	0.41	27.89
3 Days	**MEAN**	**0.00**	**0.00**	**5.95**	**0.23**	**1.16**	**0.03**	**0.00**	**0.00**	**7.11**	**0.26**	**96.64**	**3.36**
	STD	0.00	0.00	2.38	0.18	0.23	0.05	0.00	0.00	2.34	0.19	1.97	1.97
	SEM	0.00	0.00	0.84	0.06	0.08	0.02	0.00	0.00	0.83	0.07	0.70	0.70
	%CV	N/A	N/A	14.11	28.18	7.08	55.01	N/A	N/A	11.63	25.20	0.72	20.70
5 Days	**MEAN**	**0.00**	**0.00**	**1.96**	**28.95**	**0.37**	**3.13**	**0.04**	**0.05**	**2.36**	**32.13**	**8.81**	**91.19**
	STD	0.00	0.00	2.07	16.77	0.29	1.24	0.10	0.14	2.17	16.91	7.69	7.69
	SEM	0.00	0.00	0.73	5.93	0.10	0.44	0.04	0.05	0.77	5.98	2.72	2.72
	%CV	N/A	N/A	37.41	20.48	27.64	14.05	100.00	100.00	32.51	18.61	30.83	2.98
7 Days	**MEAN**	**0.00**	**0.00**	**0.50**	**43.88**	**0.24**	**5.31**	**0.00**	**0.50**	**0.74**	**49.69**	**1.42**	**98.58**
	STD	0.00	0.00	0.46	18.66	0.16	2.07	0.00	0.40	0.44	18.94	0.60	0.60
	SEM	0.00	0.00	0.16	6.60	0.06	0.73	0.00	0.14	0.16	6.70	0.21	0.21
	%CV	N/A	N/A	32.50	15.04	23.89	13.80	N/A	28.05	20.90	13.48	14.86	0.21
14 Days	**MEAN**	**0.00**	**0.00**	**0.00**	**27.15**	**0.00**	**2.42**	**0.00**	**0.17**	**0.00**	**29.74**	**0.00**	**100.00**
	STD	0.00	0.00	0.00	7.89	0.00	3.01	0.00	0.31	0.00	9.79	0.00	0.00
	SEM	0.00	0.00	0.00	2.79	0.00	1.06	0.00	0.11	0.00	3.46	0.00	0.00
	%CV	N/A	N/A	N/A	10.28	N/A	44.03	N/A	63.51	N/A	11.64	N/A	0.00
28 Days	**MEAN**	**0.00**	**4.08**	**0.14**	**30.65**	**0.03**	**3.73**	**0.00**	**0.00**	**0.17**	**38.47**	**0.46**	**99.54**
	STD	0.00	10.01	0.18	11.34	0.07	2.37	0.00	0.00	0.23	10.11	0.66	0.66
	SEM	0.00	3.54	0.06	4.01	0.03	0.84	0.00	0.00	0.08	3.57	0.23	0.23
	%CV	N/A	86.61	46.36	13.08	100.00	22.48	N/A	N/A	48.25	9.29	51.17	0.24
42 Days	**MEAN**	**0.00**	**0.00**	**0.00**	**32.03**	**0.00**	**3.58**	**0.00**	**0.12**	**0.00**	**35.73**	**0.00**	**100.00**
	STD	0.00	0.00	0.00	19.41	0.00	2.13	0.00	0.17	0.00	19.41	0.00	0.00
	SEM	0.00	0.00	0.00	6.86	0.00	0.75	0.00	0.06	0.00	6.86	0.00	0.00
	%CV	N/A	N/A	N/A	21.42	N/A	21.05	N/A	52.67	N/A	19.21	N/A	0.00

Table 4.3: Mean volumes and percentages of fibrin and collagen, together with SD, SEM and %CV.

Injury Time	P VALUE			
	Total Tissue Generation		Percentage	
	Fibrin	Collagen	Fibrin	Collagen
1 Hour - 4 Hours	0.7188	1.0000	1.0000	1.0000
4 Hours - 8 Hours	0.1744	1.0000	1.0000	1.0000
8 Hours - 16 Hours	0.7210*	1.0000	1.0000	1.0000
16 Hours - 24 Hours	0.0030*	0.0019*	0.0019*	0.0019*
24 Hours - 3 Days	0.070**	0.9093	0.0830*	0.0830*
3 Days - 5 Days	0.0009	0.0002*	0.0002**	0.0002**
5 Days - 7 Days	0.0207*	0.0707	0.0148*	0.0148*
7 Days - 14 Days	0.0002*	0.0192	0.0002*	0.0002*
14 Days - 28 Days	0.1050*	0.1011	0.1050*	0.1050*
28 Days - 42 Days	0.1050*	0.7283	0.1050*	0.1050*

Table 4.4: T Test analysis of fibrin and collagen volumes and percentages.
* = Normality Test Failed; Mann-Whitney Rank Sum Test Ran.
** = Equal Variance Test Failed; Mann-Whitney Rank Sum Test Ran.

Injury Time	P VALUE		
	PSA vs PTG	PSA vs UTG	PTG vs UTG
1 Hour	-	-	-
4 Hours	-	-	-
8 Hours	-	-	-
16 Hours	-	-	-
24 Hours	-	-	0.00186*
3 Days	-	-	0.234**
5 Days	-	-	0.5945
7 Days	-	-	0.0104**
14 Days	-	-	-
28 Days	0.400*	0.889*	0.328*
42 Days	-	-	-

Table 4.5: T Test analysis comparing percentages of fibrin and collagen between tissue zones.
* = Normality Test Failed; Mann-Whitney Rank Sum Test Ran.
** = Equal Variance Test Failed; Mann-Whitney Rank Sum Test Ran.

	Injury Time		Post Surgical Adhesion					
			Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total Number
Number Density	**28 Days**	**Mean**	**0.00**	**12500.00**	**133333.33**	**308333.33**	**0.00**	**454166.67**
		STD	N/A	N/A	N/A	N/A	N/A	N/A
		SEM	N/A	N/A	N/A	N/A	N/A	N/A
		CV%	N/A	N/A	N/A	N/A	N/A	N/A
		%	0.00	2.75	29.36	67.89	0.00	100.00
Number Total	"	**Mean**	**0.00**	**422497.56**	**4506640.63**	**10421606.45**	**0.00**	**15350744.63**
		STD	N/A	N/A	N/A	N/A	N/A	N/A
		SEM	N/A	N/A	N/A	N/A	N/A	N/A
		CV%	N/A	N/A	N/A	N/A	N/A	N/A
		%	0.00	2.75	29.36	67.89	0.00	100.00

Table 4.6: Mean number density and total for cell types within PSAs.

Injury Time		Peritoneal Tissue Generation						Uterine Horn Tissue Generation						Fibrin Sealant Associated Cell Concentration					
		Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.
1 Hour	Mean	4924.91	6142.33	8709.11	0.00	3403.08	23179.43	0.00	23319.62	17832.65	0.00	49382.72	90534.98	-	-	-	-	-	-
	STD	5710.34	10295.82	7918.41	0.00	3741.55	18157.79	0.00	20299.93	18556.58	0.00	85533.37	51399.16	-	-	-	-	-	-
	SEM	2018.91	3640.12	2799.58	0.00	1322.84	6419.75	0.00	11720.17	10713.65	0.00	49382.72	29675.32	-	-	-	-	-	-
	CV%	40.99	59.26	32.15	N/A	38.87	27.70	N/A	50.26	60.08	N/A	100.00	32.78	-	-	-	-	-	-
	%	21.25	26.50	37.57	0.00	14.68	100.00	0.00	25.76	19.70	0.00	54.55	100.00	-	-	-	-	-	-
4 Hours	Mean	14534.09	81086.75	11524.15	0.00	4060.81	111205.79	10934.74	103556.73	21575.54	0.00	16813.64	152880.66	-	-	-	-	-	-
	STD	11431.33	35050.37	9056.56	0.00	3878.39	47054.75	19106.44	100436.31	16260.54	0.00	27560.17	112375.38	-	-	-	-	-	-
	SEM	4041.59	12392.18	3201.98	0.00	1371.22	16636.37	7221.55	37961.36	6145.91	0.00	10416.76	42473.90	-	-	-	-	-	-
	CV%	27.81	15.28	27.78	N/A	33.77	14.96	66.04	36.66	28.49	N/A	61.95	27.78	-	-	-	-	-	-
	%	13.07	72.92	10.36	0.00	3.65	100.00	7.15	67.74	14.11	0.00	11.00	100.00	-	-	-	-	-	-
8 Hours	Mean	7308.92	160574.75	17540.94	0.00	3956.81	189381.42	7485.60	150107.95	9617.53	0.00	10035.95	177247.03	-	-	-	-	-	-
	STD	10211.01	86453.93	11199.36	0.00	3863.77	84553.60	9351.80	70668.60	8273.05	0.00	9952.05	75975.27	-	-	-	-	-	-
	SEM	3610.14	30566.08	3959.57	0.00	1366.05	29894.21	3534.65	26710.22	3126.92	0.00	3761.52	28715.95	-	-	-	-	-	-
	CV%	49.39	19.04	22.57	N/A	34.52	15.79	47.22	17.79	32.51	N/A	37.48	16.20	-	-	-	-	-	-
	%	3.86	84.79	9.26	0.00	2.09	100.00	4.22	84.69	5.43	0.00	5.66	100.00	-	-	-	-	-	-
16 Hours	Mean	0.00	241662.61	37320.32	15344.66	2189.41	296517.01	0.00	164427.99	35099.56	29656.86	3335.18	232519.60	-	-	-	-	-	-
	STD	0.00	64758.06	21341.58	5433.65	3243.57	73946.58	0.00	48920.03	21612.08	13102.93	3281.19	43474.59	-	-	-	-	-	-
	SEM	0.00	22895.43	7545.39	1921.09	1146.78	26144.06	0.00	17295.84	7641.02	4632.58	1160.08	15370.59	-	-	-	-	-	-
	CV%	N/A	9.47	20.22	12.52	52.38	8.82	N/A	10.52	21.77	15.62	34.78	6.61	-	-	-	-	-	-
	%	0.00	81.50	12.59	5.17	0.74	100.00	0.00	70.72	15.10	12.75	1.43	100.00	-	-	-	-	-	-
1 Day	Mean	231.48	196782.82	49715.12	16033.14	881.56	263644.13	440.92	127503.40	65311.01	39089.00	2087.05	234431.37	-	-	-	-	-	-
	STD	654.73	61059.76	23912.69	8780.23	1307.21	79422.34	1247.10	11961.63	25838.97	5130.22	1776.06	35658.40	-	-	-	-	-	-
	SEM	231.48	21587.88	8454.41	3104.28	462.17	28080.04	440.92	4229.08	9135.45	1813.81	627.93	12607.15	-	-	-	-	-	-
	CV%	100.00	10.97	17.01	19.36	52.43	10.65	100.00	3.32	13.99	4.64	30.09	5.38	-	-	-	-	-	-
	%	0.09	74.64	18.86	6.08	0.33	100.00	0.19	54.39	27.86	16.67	0.89	100.00	-	-	-	-	-	-
3 Days	Mean	803.76	36393.68	137353.04	115470.80	0.00	290021.28	0.00	135831.86	164442.48	160155.79	0.00	460430.14	1736.11	329890.08	656092.69	216360.55	0.00	1204079.43
	STD	1564.12	29265.83	51413.55	42364.77	0.00	93146.79	0.00	228419.95	84456.82	85408.74	0.00	241039.46	4910.46	185095.66	113461.61	51473.86	0.00	143210.69
	SEM	553.00	10347.03	18177.44	14978.21	0.00	32932.36	0.00	93252.06	34479.35	34867.97	0.00	98403.95	1736.11	65441.20	40114.74	18198.76	0.00	50632.62
	CV%	68.80	28.43	13.23	12.97	N/A	11.36	N/A	68.65	20.97	21.77	N/A	21.37	100.00	19.84	6.11	8.41	N/A	4.21
	%	0.28	12.55	47.36	39.81	0.00	100.00	0.00	29.50	35.71	34.78	0.00	100.00	0.14	27.40	54.49	17.97	0.00	100.00
5 Days	Mean	429.55	9169.92	201418.67	246999.51	1488.10	459505.75	1302.08	9242.24	161930.24	343100.65	744.05	516319.26	1135.54	11974.43	406045.04	261684.42	4659.43	685498.86
	STD	1214.96	5469.34	46531.43	39493.31	4208.97	85144.86	3682.85	14076.15	41833.89	66729.39	2104.48	95148.86	2132.55	12014.02	82763.89	83377.38	5305.39	145070.32
	SEM	429.55	1933.70	16451.35	13962.99	1488.10	30103.26	1302.08	4976.67	14790.51	23592.40	744.05	33640.20	753.97	4247.60	29261.45	29478.36	1875.74	51290.10
	CV%	100.00	21.09	8.17	5.65	100.00	6.55	100.00	53.85	9.13	6.88	100.00	6.52	66.40	35.47	7.21	11.26	40.26	7.48
	%	0.09	2.00	43.83	53.75	0.32	100.00	0.25	1.79	31.36	66.45	0.14	100.00	0.17	1.75	59.23	38.17	0.68	100.00
7 Days	Mean	165.34	32064.47	109124.48	152644.39	330.69	294329.37	0.00	7559.09	135138.14	169309.62	0.00	312006.85	0.00	483655.75	479985.12	307093.25	0.00	1270734.13
	STD	467.66	23118.36	28522.99	39052.20	935.33	56814.55	0.00	4467.31	42231.90	36878.24	0.00	71654.71	0.00	155263.95	123414.13	37746.68	0.00	102188.02
	SEM	165.34	8173.58	10084.40	13807.04	330.69	20086.98	0.00	1688.49	15962.16	13938.66	0.00	27082.94	0.00	54894.10	43633.48	13345.47	0.00	36128.92
	CV%	100.00	25.49	9.24	9.05	100.00	6.82	N/A	22.34	11.81	8.23	N/A	8.68	N/A	11.35	9.09	4.35	N/A	2.84
	%	0.06	10.89	37.08	51.86	0.11	100.00	0.00	2.42	43.31	54.26	0.00	100.00	0.00	38.06	37.77	24.17	0.00	100.00
14 Days	Mean	14661.42	42565.59	102746.12	251148.57	3559.48	414681.19	17390.15	0.00	84315.50	248464.24	2351.56	352521.45	-	-	-	-	-	-
	STD	37063.03	19605.07	68950.25	96524.60	4823.27	187316.49	41437.12	0.00	56846.81	94460.14	6221.64	173602.81	-	-	-	-	-	-
	SEM	13103.76	6931.44	24377.59	34126.60	1705.28	66226.38	15661.76	0.00	21486.07	35702.58	2351.56	65615.69	-	-	-	-	-	-
	CV%	89.38	16.28	23.73	13.59	47.91	15.97	90.06	N/A	25.48	14.37	100.00	18.61	-	-	-	-	-	-
	%	3.54	10.26	24.78	60.56	0.86	100.00	4.93	0.00	23.92	70.48	0.67	100.00	-	-	-	-	-	-
28 Days	Mean	0.00	49170.81	108376.09	167361.33	0.00	324908.23	0.00	4900.34	103598.41	203691.23	0.00	312189.98	-	-	-	-	-	-
	STD	0.00	47264.40	40079.48	54840.20	0.00	124065.94	0.00	8348.55	31478.46	73419.30	0.00	73570.70	-	-	-	-	-	-
	SEM	0.00	16710.49	14170.24	19388.94	0.00	43863.93	0.00	2951.66	11129.32	25957.64	0.00	26011.17	-	-	-	-	-	-
	CV%	N/A	33.98	13.08	11.59	N/A	13.50	N/A	60.23	10.74	12.74	N/A	8.33	-	-	-	-	-	-
	%	0.00	15.13	33.36	51.51	0.00	100.00	0.00	1.57	33.18	65.25	0.00	100.00	-	-	-	-	-	-
42 Days	Mean	6153.53	2816.47	31076.70	168578.55	1496.76	210122.01	8950.62	641.24	44446.84	226704.97	253.68	280997.35	-	-	-	-	-	-
	STD	9435.29	3370.62	10792.91	67824.20	1866.47	76726.36	20781.75	1447.91	11797.40	107621.81	717.51	118142.24	-	-	-	-	-	-
	SEM	3335.88	1191.69	3815.87	23979.48	659.90	27126.86	7347.46	511.92	4171.01	38050.06	253.68	41769.59	-	-	-	-	-	-
	CV%	54.21	42.31	12.28	14.22	44.09	12.91	82.09	79.83	9.38	16.78	100.00	14.86	-	-	-	-	-	-
	%	2.93	1.34	14.79	80.23	0.71	100.00	3.19	0.23	15.82	80.68	0.09	100.00	-	-	-	-	-	-

Table 4.7: Number densities for basophilic cells, eosinophilic cells, macrophages, fibroblasts, lymphocytes and totals within PTG, UTG and fibrin sealant associated cell concentration.

Injury Time	P Value Peritoneal Tissue Generation						P Value Uterine Horn Tissue Generation						P Value Fibrin Sealant Associated Cell Concentration					
	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.
1-4 Hours	0.0650**	<0.0001	0.5188	1.0000	0.7351	0.0002	0.0621	0.2209	0.7560	1.0000	0.3634	0.3956	-	-	-	-	-	-
4-8 Hours	0.1950*	0.0303	0.2571	1.0000	0.9579	0.0384	0.9020*	0.3357	0.1085	1.0000	0.4560**	0.6431	-	-	-	-	-	-
8-16 Hours	0.0104*	0.0520	0.0359	0.0002*	0.3820*	0.0173	0.0721*	0.6940**	0.0118	0.0022*	0.0944	0.2320**	-	-	-	-	-	-
16 Hours-1 Day	0.7210*	0.1757	0.2925	0.7980*	0.7210*	0.4060	0.7210*	0.1950**	0.0237	0.0281*	0.3601	0.9247	-	-	-	-	-	-
1-3 Days	0.6450*	<0.0001	0.0006	0.0002*	0.2340*	0.5520	0.7550*	0.0426*	0.0080**	0.0007*	0.0200*	0.0080*	-	-	-	-	-	-
3-5 Days	0.7210*	0.0006*	0.0204	<0.0001	0.7210*	0.0020	0.7550*	0.0593*	0.9425	0.0007	0.7550*	0.0003	0.7980*	0.0002*	0.0002	0.2118	0.0379*	<0.0001
5-7 Days	0.9590*	0.0003*	0.0003	0.0003	0.9590*	0.0004	0.6940*	0.6130*	0.2398	<0.0001	0.6940*	0.0005	0.4420*	0.0002**	0.1811	0.1823	0.0379*	<0.0001
7-14 Days	0.1610*	0.3438	0.3820*	0.0181	0.0650*	0.0499*	0.2090*	0.0041*	0.0819	0.0612	0.7100*	0.5787	-	-	-	-	-	-
14-28 Days	0.1050*	0.7205	0.5050*	0.0509	0.0379*	0.2774	0.1890*	0.2320*	0.4226	0.3207	0.6940*	0.8670*	-	-	-	-	-	-
28-42 Days	0.0379*	0.0006*	0.0002**	0.9691	0.1050*	0.0430	0.4420*	0.5740*	0.0011**	0.6251	0.7210*	0.5364	-	-	-	-	-	-

Table 4.8: T Test analysis of number density for each cell types between concurrent time points.

Injury Time	P Value					
	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.
1 Hour	0.1825	0.0866	0.4970**	1.0000	0.9210*	0.0242**
4 Hours	0.6601	0.9550**	0.1559	1.0000	1.0000	0.7790**
8 Hours	0.9550*	0.8033	0.1481	1.0000	0.1331	0.7760
16 Hours	1.0000	0.0175	0.8391	0.0104*	0.3820*	0.0533
1 Day	0.9590*	0.0104**	0.2307	0.0002*	0.1610*	0.3587
3 Days	0.4910*	0.7550*	0.4697	0.4910*	1.0000	0.0899
5 Days	0.9590*	0.9894	0.0959	0.0035	0.9590*	0.2288
7 Days	0.6940*	0.0003*	0.1805	0.4129	0.6940*	0.6031
14 Days	1.0000	0.0003*	0.4630*	0.9575	0.2320*	0.2810*
28 Days	1.0000	0.0206*	0.7948	0.2810	1.0000	0.8067
42 Days	0.3820*	0.1610*	0.0330	0.2171	0.1950*	0.1766

Table 4.9: T Test analysis comparing PTG and UTG with regard to number density for each cell type.

Injury Time		Peritoneal Tissue Generation						Uterine Horn Tissue Generation						Fibrin Sealant Associated Cell Concentration					
		Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.
1 Hour	Mean	24350.64	26109.13	34171.62	0.00	10127.41	94758.80	0.00	16246.91	13996.39	0.00	41558.16	71801.46	-	-	-	-	-	-
	STD	32802.62	40994.49	31295.63	0.00	10076.28	83961.03	0.00	17778.33	18702.63	0.00	71980.84	52222.56	-	-	-	-	-	-
	SEM	11597.48	14493.74	11064.68	0.00	3562.50	29684.71	0.00	10264.32	10797.97	0.00	41558.16	30150.71	-	-	-	-	-	-
	CV%	47.63	55.51	32.38	N/A	35.18	31.33	N/A	63.18	77.15	N/A	100.00	41.99	-	-	-	-	-	-
	%	25.70	27.55	36.06	0.00	10.69	100.00	0.00	22.63	19.49	0.00	57.88	100.00	-	-	-	-	-	-
4 Hours	Mean	123686.69	642413.04	83261.21	0.00	27281.57	876642.50	1702.81	54036.32	25429.95	0.00	19122.20	100291.28	-	-	-	-	-	-
	STD	114325.87	350144.87	74420.73	0.00	26669.81	467957.27	2933.81	53939.66	47943.35	0.00	45680.77	56979.35	-	-	-	-	-	-
	SEM	40420.30	123794.91	26311.70	0.00	9429.20	165447.88	1108.87	20387.27	18120.88	0.00	17265.71	21536.17	-	-	-	-	-	-
	CV%	32.68	19.27	31.60	N/A	34.56	18.87	65.12	37.73	71.26	N/A	90.29	21.47	-	-	-	-	-	-
	%	14.11	73.28	9.50	0.00	3.11	100.00	1.70	53.88	25.36	0.00	19.07	100.00	-	-	-	-	-	-
8 Hours	Mean	112433.32	2054102.63	246674.35	0.00	66411.06	2479621.36	25426.61	531017.49	32019.39	0.00	25033.33	613496.83	-	-	-	-	-	-
	STD	135491.59	874125.85	151101.22	0.00	79943.84	973074.38	26908.03	362394.15	30333.53	0.00	26693.83	392911.52	-	-	-	-	-	-
	SEM	47903.51	309050.16	53422.35	0.00	28264.42	344033.75	10170.28	136972.11	11465.00	0.00	10089.32	148506.60	-	-	-	-	-	-
	CV%	42.61	15.05	21.66	N/A	42.56	13.87	40.00	25.79	35.81	N/A	40.30	24.21	-	-	-	-	-	-
	%	4.53	82.84	9.95	0.00	2.68	100.00	4.14	86.56	5.22	0.00	4.08	100.00	-	-	-	-	-	-
16 Hours	Mean	0.00	3022093.14	444111.83	177949.76	25751.54	3669906.26	0.00	964591.18	223355.31	182604.09	20443.33	1390993.91	-	-	-	-	-	-
	STD	0.00	1716980.27	286366.03	69508.19	38149.86	1972474.47	0.00	348499.95	170712.17	111617.14	18265.59	478745.35	-	-	-	-	-	-
	SEM	0.00	607044.20	101245.68	24574.85	13488.01	697375.04	0.00	123213.34	60355.87	39462.62	6457.86	169262.04	-	-	-	-	-	-
	CV%	N/A	20.09	22.80	13.81	52.38	19.00	N/A	12.77	27.02	21.61	31.59	12.17	-	-	-	-	-	-
	%	0.00	82.35	12.10	4.85	0.70	100.00	0.00	69.35	16.06	13.13	1.47	100.00	-	-	-	-	-	-
1 Day	Mean	6239.15	5993510.72	1471043.46	468545.03	34121.71	7973460.08	1416.62	1620792.89	778814.59	522457.14	25097.24	2948578.47	-	-	-	-	-	-
	STD	17646.98	2752498.76	778339.74	260843.42	54494.34	3572878.20	4006.80	724838.34	426833.11	281718.26	30922.92	1323864.62	-	-	-	-	-	-
	SEM	6239.15	973155.27	275184.65	92222.08	19266.66	1263203.20	1416.62	256269.05	150908.29	99602.45	10932.90	468056.83	-	-	-	-	-	-
	CV%	100.00	16.24	18.71	19.68	56.46	15.84	100.00	15.81	19.38	19.06	43.56	15.87	-	-	-	-	-	-
	%	0.08	75.17	18.45	5.88	0.43	100.00	0.05	54.97	26.41	17.72	0.85	100.00	-	-	-	-	-	-
3 Days	Mean	27324.16	1025740.55	3815827.08	3192380.91	0.00	8061272.70	0.00	332456.65	529924.76	485021.56	0.00	1347402.97	111490.89	12712063.44	23954541.08	7962306.63	0.00	44740402.04
	STD	54896.04	884237.65	1347464.67	1026665.01	0.00	2357301.47	0.00	435185.67	388604.34	320996.36	0.00	766005.99	315343.84	9667610.98	10979633.43	3821104.20	0.00	21636224.70
	SEM	19408.68	312625.22	476400.70	362980.89	0.00	833431.93	0.00	177663.80	158647.06	131046.21	0.00	312720.64	111490.89	3418016.64	3881886.63	1350964.34	0.00	7649560.60
	CV%	71.03	30.48	12.48	11.37	N/A	10.34	N/A	53.44	29.94	27.02	N/A	23.21	100.00	26.89	16.21	16.97	N/A	17.10
	%	0.34	12.72	47.34	39.60	0.00	100.00	0.00	24.67	39.33	36.00	0.00	100.00	0.25	28.41	53.54	17.80	0.00	100.00
5 Days	Mean	7382.95	667856.08	14641750.66	17584521.11	170026.51	33071537.31	7692.97	42051.71	892246.32	1868441.69	1442.14	2811874.83	49178.91	744295.01	21378869.98	12946905.11	145246.13	35264495.15
	STD	20882.13	481569.95	7718135.26	8226335.71	480907.59	16451935.33	21759.01	63589.35	436196.74	873626.88	4078.98	1279756.15	105643.36	850106.67	20271447.52	11464340.63	161710.08	32171466.98
	SEM	7382.95	170260.69	2728772.89	2908448.88	170026.51	5816637.52	7692.97	22482.23	154218.84	308873.75	1442.14	452462.13	37350.57	300558.10	7167039.00	4053256.50	57173.15	11374331.23
	CV%	100.00	25.49	18.64	16.54	100.00	17.59	100.00	53.46	17.28	16.53	100.00	16.09	75.95	40.38	33.52	31.31	39.36	32.25
	%	0.02	2.02	44.27	53.17	0.51	100.00	0.27	1.50	31.73	66.45	0.05	100.00	0.14	2.11	60.62	36.71	0.41	100.00
7 Days	Mean	10602.68	2339293.59	8584626.80	11692974.95	21205.36	22648703.38	0.00	52088.47	959986.77	1195508.44	0.00	2207583.68	0.00	17601996.72	16266550.02	10637236.00	0.00	44505782.75
	STD	29988.90	1656223.14	4785010.86	5515928.28	59977.81	11207973.74	0.00	32789.67	579658.95	559065.28	0.00	1126570.23	0.00	8058432.59	4472530.02	2541572.27	0.00	11576893.17
	SEM	10602.68	585563.31	1691756.81	1950175.15	21205.36	3962617.12	0.00	12393.33	219090.49	211306.81	0.00	425803.52	0.00	2849086.17	1581278.15	898581.49	0.00	4093049.83
	CV%	100.00	25.03	19.71	16.68	100.00	17.50	N/A	23.79	22.82	17.68	N/A	19.29	N/A	16.19	9.72	8.45	N/A	9.20
	%	0.05	10.33	37.90	51.63	0.09	100.00	0.00	2.36	43.49	54.15	0.00	100.00	0.00	39.55	36.55	23.90	0.00	100.00
14 Days	Mean	1022463.51	2465000.31	6380251.03	15194050.28	212972.20	25274737.33	8436.84	0.00	213681.13	700467.40	1988.72	924574.09	-	-	-	-	-	-
	STD	2677450.08	1644270.97	5554967.29	9285521.36	301774.63	16968467.30	15861.82	0.00	193356.23	739609.68	5261.66	912973.74	-	-	-	-	-	-
	SEM	946621.55	581337.58	1963977.52	3282927.56	106693.45	5999259.15	5995.20	0.00	73081.78	279546.18	1988.72	345071.64	-	-	-	-	-	-
	CV%	92.58	23.58	30.78	21.61	50.10	23.74	71.06	N/A	34.20	39.91	100.00	37.32	-	-	-	-	-	-
	%	4.05	9.75	25.24	60.12	0.84	100.00	0.91	0.00	23.11	75.76	0.22	100.00	-	-	-	-	-	-
28 Days	Mean	0.00	2210137.10	4739159.49	6890300.64	0.00	13839597.23	0.00	23375.30	545968.81	1195057.34	0.00	1764401.45	-	-	-	-	-	-
	STD	0.00	2313038.77	2179045.99	2520040.69	0.00	6508867.86	0.00	34183.50	405709.53	1097672.51	0.00	1448285.31	-	-	-	-	-	-
	SEM	0.00	817782.70	770409.10	890968.93	0.00	2301232.30	0.00	12085.69	143439.98	388085.84	0.00	512046.18	-	-	-	-	-	-
	CV%	N/A	37.00	16.26	12.93	N/A	16.63	N/A	51.70	26.27	32.47	N/A	29.02	-	-	-	-	-	-
	%	0.00	15.97	34.24	49.79	0.00	100.00	0.00	1.32	30.94	67.73	0.00	100.00	-	-	-	-	-	-
42 Days	Mean	140028.68	122421.36	1186493.62	6197540.24	52200.41	7698684.31	22741.77	3224.19	164800.48	717392.40	2771.20	910930.04	-	-	-	-	-	-
	STD	196694.68	195930.09	646253.57	3660985.03	67584.85	4179487.82	58737.94	6048.09	110217.23	352477.77	7838.14	449887.43	-	-	-	-	-	-
	SEM	69542.07	69271.75	228485.14	1294353.67	23894.85	1477672.09	20767.00	2138.32	38967.68	124619.71	2771.20	159059.23	-	-	-	-	-	-
	CV%	49.66	56.58	19.26	20.88	45.78	19.19	91.32	66.32	23.65	17.37	100.00	17.46	-	-	-	-	-	-
	%	1.82	1.59	15.41	80.50	0.68	100.00	2.50	0.35	18.09	78.75	0.30	100.00	-	-	-	-	-	-

Table 4.10: Number totals for basophilic cells, eosinophilic cells, macrophages, fibroblasts, lymphocytes and totals within PTG, UTG and fibrin sealant associated cell concentration.

Injury Time	P Value Peritoneal Tissue Generation						P Value Uterine Horn Tissue Generation						P Value Fibrin Sealant Associated Cell Concentration					
	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.
1-4 Hours	0.0499**	0.0002*	0.1075	1.0000	0.1109	0.0002**	0.5170*	0.2827	1.0000*	1.0000	0.8330*	0.4807	-	-	-	-	-	-
4-8 Hours	0.7980**	0.0003**	0.0158	1.0000	0.7210**	0.0006**	0.1650*	0.0175**	0.3830*	1.0000	0.1650*	0.0175**	-	-	-	-	-	-
8-16 Hours	0.0104*	0.1772	0.1066	0.0002*	0.2790*	0.1481	0.0721*	0.0346	0.0006**	0.0022*	0.7005	0.0047	-	-	-	-	-	-
16 Hours-1 Day	0.7210*	0.0214	0.0030**	0.0087	0.9590*	0.0099	0.7210*	0.0368	0.0030**	0.0207**	0.7195	0.0379**	-	-	-	-	-	-
1-3 Days	0.6450*	0.0003**	0.0008	0.0002**	0.2340*	0.9545	0.7550*	0.0025	0.2741	0.8117	0.0200*	0.0185	-	-	-	-	-	-
3-5 Days	0.6450*	0.3318	0.0007*	0.0007*	0.7210*	0.0030**	0.7550*	0.1080*	0.1260	0.0030	0.7550*	0.0248	0.7980*	0.0002*	0.7567	0.2790*	0.0379*	0.5007
5-7 Days	0.9590*	0.0159	0.0801	0.1146	0.9590*	0.1608	0.6940*	0.7137	0.8005	0.1046	0.6940*	0.3527	0.4420*	0.0002*	0.9590*	0.8780*	0.0379*	0.1050*
7-14 Days	0.1610*	0.8811	0.1950*	0.3747	0.0830*	0.8780*	0.2090*	0.0041*	0.0012*	0.0379*	0.7100*	0.0175*	-	-	-	-	-	-
14-28 Days	0.105*	0.2790*	0.4496	0.0207*	0.0379*	0.9680	0.1890*	0.2320*	0.0701	0.3325	0.6940*	0.2100	-	-	-	-	-	-
28-42 Days	0.0379*	0.0006*	0.0030**	0.6660	0.1050*	0.0414	0.4420*	0.5050*	0.0207**	0.2608	0.7210*	0.2340**	-	-	-	-	-	-

Table 4.11: T Test analysis of number totals for each cell types between concurrent time points.

Injury Time	P Value					
	Basophilic Cells	Eosinophilic Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.
1 Hour	0.2452	0.7038	0.3305	1.0000	0.2183	0.6741
4 Hours	0.0040*	0.0004**	0.0205*	1.0000	0.1520*	0.0004**
8 Hours	0.1202	0.0004**	0.0022**	1.0000	0.2155	0.0003**
16 Hours	1.0000	0.0002**	0.0821	0.9217	0.9590*	0.0006**
1 Day	0.9590*	0.0030**	0.0446	0.6972	0.6450*	0.0003**
3 Days	0.4910*	0.04260*	0.0007*	0.0007*	1.0000	0.0007**
5 Days	0.9590*	0.0070*	0.0002*	0.0002*	0.9590*	0.0002**
7 Days	0.6940*	0.0003*	0.0003*	0.0003*	0.6940*	0.0003*
14 Days	0.3970*	0.0003*	0.0003*	0.0003*	0.0721*	0.0003*
28 Days	1.0000	0.0002*	0.0003**	<0.0001	1.0000	0.0002**
42 Days	0.1610*	0.0830*	0.0003**	0.0002**	0.1610*	0.0002*

Table 4.12: T Test analysis comparing PTG and UTG with regard to number totals for each cell type.

Cell Type	x10^9/l Mean	SD	SEM	CV%
Blood Sample 1				
RBCs (x10^3/l)	5.60	0.50	0.08	1.49
Platelets	252.50	123.09	19.00	7.52
WBCs	4.44	1.08	0.17	3.83
Basophilic Cells	0.15	0.11	0.03	22.63
Lymphocytes	2.28	0.80	0.24	10.61
Monocytes	0.54	0.25	0.08	13.96
Eosinophilic Cells	1.68	0.80	0.24	14.33
Blood Sample 2				
RBCs (x10^3/l)	5.16	0.53	0.09	1.74
Platelets	266.00	105.36	17.32	6.51
WBCs	4.06	1.32	0.22	5.42
Basophilic Cells	0.10	1.13	0.04	39.27
Lymphocytes	1.64	0.83	0.25	15.15
Monocytes	0.42	0.21	0.06	14.91
Eosinophilic Cells	1.91	0.88	0.26	13.81
Blood Sample 3				
RBCs (x10^3/l)	4.83	0.93	0.15	3.18
Platelets	284.97	153.70	25.27	8.87
WBCs	4.26	1.65	0.27	6.37
Basophilic Cells	0.12	0.11	0.02	14.22
Lymphocytes	1.39	0.65	0.11	7.64
Monocytes	0.28	0.17	0.03	9.70
Eosinophilic Cells	2.47	1.39	0.23	9.26

Table 4.13: Mean numbers of cell types for all blood samples, together with the SEM, SD, SEM and CV%.

	Basophilic Cells	Lymphocytes	Macrophages	Eosinophilic Cells	WBCs	RBCs	Platelets
Blood sample 1 vs 2	0.3278	0.0809	0.2158	0.5141	0.1453	0.0034	0.6192
Blood sample 1 vs 3	0.5000*	0.0004	0.0003	0.0781	0.3070**	<0.0001*	0.1471
Blood sample 2 vs 3	0.2590*	0.2867	0.037	0.2164	0.5334	0.7410*	0.2902

Table 4.14: T test analysis of cell types for blood samples.
* = normality test failed; Mann Whitney rank sum test ran.
** = equal variance failed; Mann Whitney rank sum test ran.

Injury Time		Number of White Blood Cell ($x10^9/l$)					RBCs ($x10^{12}/l$)	Platelets ($x10^9/l$)
		Basophilic Cells	Lymphocytes	Monocytes	Eosinophilic Cells	Total		
1 Hour	MEAN	0.06	1.22	0.22	1.94	3.45	5.42	316.00
	STD	0.05	0.31	0.14	1.10	0.83	0.41	64.92
	SEM	0.02	0.15	0.07	0.55	0.42	0.21	32.46
	CV%	39.90	12.60	31.59	28.43	12.10	3.80	10.27
4 Hours	MEAN	0.11	1.34	0.16	3.26	4.87	5.62	438.50
	STD	0.10	0.37	0.02	0.69	0.50	0.90	422.14
	SEM	0.06	0.21	0.01	0.40	0.29	0.52	298.50
	CV%	50.13	15.94	5.76	12.28	5.97	9.27	68.07
8 Hours	MEAN	0.15	0.90	0.33	4.66	6.05	4.97	180.00
	STD	0.20	0.77	0.21	1.39	2.10	0.54	128.01
	SEM	0.10	0.39	0.11	0.70	1.05	0.27	73.91
	CV%	66.32	42.85	32.00	14.94	17.32	5.39	41.06
16 Hours	MEAN	0.16	1.80	0.16	2.75	4.87	4.61	391.67
	STD	0.15	0.45	0.04	0.20	0.78	1.00	156.11
	SEM	0.09	0.26	0.02	0.12	0.45	0.58	90.13
	CV%	56.32	14.36	14.89	4.29	9.21	12.53	23.01
1 Day	MEAN	0.23	1.89	0.37	2.34	4.80	4.82	187.00
	STD	0.09	0.81	0.20	0.80	0.77	0.33	59.16
	SEM	0.04	0.40	0.10	0.40	0.39	0.16	29.58
	CV%	18.33	21.37	27.09	17.07	8.07	3.40	15.82
3 Days	MEAN	0.05	1.38	0.18	1.46	3.08	4.71	359.75
	STD	0.01	0.73	0.09	0.29	0.78	0.30	68.66
	SEM	0.01	0.37	0.04	0.14	0.39	0.15	34.33
	CV%	10.06	26.65	23.40	9.85	12.76	3.16	9.54
5 Days	MEAN	0.08	1.56	0.29	4.36	6.30	4.57	326.67
	STD	0.03	0.40	0.25	0.80	0.87	0.30	54.93
	SEM	0.02	0.23	0.15	0.46	0.50	0.18	31.71
	CV%	22.09	14.63	50.52	10.60	7.99	3.84	9.71
7 Days	MEAN	0.12	1.28	0.31	1.19	2.90	5.07	270.67
	STD	0.13	0.22	0.06	0.62	0.87	0.13	228.95
	SEM	0.08	0.13	0.03	0.36	0.50	0.08	132.19
	CV%	62.01	9.82	11.25	30.19	17.24	1.49	48.84
14 Days	MEAN	0.04	0.80	0.35	0.94	2.13	2.81	56.00
	STD	0.04	0.55	0.18	0.73	1.10	2.05	4.24
	SEM	0.02	0.32	0.10	0.42	0.63	1.18	2.45
	CV%	59.92	39.25	29.31	44.65	29.69	42.18	4.37
28 Days	MEAN	0.15	1.32	0.30	1.64	3.40	5.25	223.50
	STD	0.08	0.48	0.10	0.40	0.56	0.12	89.01
	SEM	0.04	0.24	0.05	0.20	0.28	0.06	44.51
	CV%	27.65	18.15	16.72	12.33	8.23	1.13	19.91
42 Days	MEAN	0.21	2.10	0.53	2.86	5.70	5.03	424.00
	STD	0.03	1.50	0.34	0.96	2.83	0.46	66.47
	SEM	0.02	1.06	0.24	0.68	2.00	0.32	47.00
	CV%	11.06	50.58	44.47	23.74	35.09	6.47	11.08

Table 4.15: Mean numbers of cell types for blood sample 3 at time points following injury, together with SD, SEM and CV%.

Sample Time	Blood Sample 1	Blood Sample 2	Treated Blood Sample 3	Peritoneal Sample 1	Treated Peritoneal Sample 3
1 Hour	-	-	3128.57	-	-
4 Hours	-	-	2931.43	-	-
8 Hours	-	-	3222.86	-	-
16 Hours	-	-	3205.71	-	-
1 Day	-	-	2151.43	-	-
3 Days	-	-	2854.29	-	3480.00
5 Days	-	-	3831.43	-	4371.43
7 Days	-	-	4637.14	-	4885.71
14 Days	-	-	2974.29	-	2674.29
28 Days	-	-	2237.14	-	1860.00
42 Days	-	-	2965.71	-	-
Mean	**4568.57**	**2997.14**	-	**1740.00**	-
STD	2877.51	696.05	-	193.95	-
SEM	1661.33	401.86	-	137.14	-
CV%	36.36	13.41		7.88	

Table 4.16: TGF-β concentrations in plasma and peritoneal fluid samples.

APPENDIX V

Injury Time	P VALUE									
	PSA	Tissue Generation			Damage Zone		Peritoneal Reaction Zone	Peritoneal Cell Concentration	Uterine Horn Haemorrhage	Total Cell Concentration
		Peritoneal	Uterine Horn	Total	Peritoneal	Uterine Horn				
1 Hour	0.4420*	0.0002*	0.3820*	0.0002*	0.3832	0.4656	0.0002*	1.0000	0.0002	1.0000
4 Hours	0.1050*	0.0070*	0.0499*	0.0002**	0.0176	0.4939	0.0002**	0.4420*	0.0002*	0.4420*
8 Hours	0.0002*	0.0513	0.1478	<0.0001	0.5050**	0.2398	<0.0001	0.4420*	0.0019*	0.4420*
16 Hours	0.0002*	0.0002	0.6114*	<0.0001	0.2559	0.1173	0.0002**	0.7980*	0.1610*	0.6450*
1 Day	0.0002*	0.8690	0.0188	0.0163	0.2672	0.0801	<0.001	0.0019*	0.0002*	0.0064
3 Days	0.0019*	0.0006**	0.5050*	0.0002*	0.0310	0.2340*	0.0002*	0.0002*	0.0379*	0.9590*
5 Days	0.0379*	0.2790**	0.0002*	0.0006	0.6661	0.0002*	0.0002**	0.7210*	0.7980*	0.0058
7 Days	0.0379*	0.0379**	0.0006*	0.0002	0.1300*	0.1950*	0.0002*	0.1050*	1.0000	0.0002*
14 Days	0.0104*	0.0003*	0.0281*	0.0002*	0.0019*	0.9590	0.0002*	0.7210*	1.0000	0.0104*
28 Days	0.0148*	0.9590*	0.5740*	0.0070*	0.0499*	0.1300*	0.0002**	0.7210*	1.0000	0.1950
42 Days	0.0019*	0.0955	0.0002*	0.0006*	0.2340*	0.1050*	0.0002**	1.0000	1.0000	1.0000

Table 5.1: T Test analysis between control and treated cases with regard to physiological volumes.
* = Normality Test Failed; Mann-Whitney Rank Sum Test Ran;
** = Equal Variance Test Failed; Mann-Whitney Rank Sum Test Ran.
Note: Total Cell Concentration : Control Peritoneal Cell Conc. vs Treated
[Peritoneal Cell Conc. + Fibrin Sealant Assoc. Cell Conc.]

Injury Time	P Value			
	Total Tissue Generation		Percentage	
	Fibrin	Collagen	Fibrin	Collagen
1 Hour	0.1976	1.0000	1.0000	1.0000
4 Hours	0.0207*	1.0000	1.0000	1.0000
8 Hours	0.0084	1.0000	1.0000	1.0000
16 Hours	0.0030*	1.0000	1.0000	1.0000
1 Day	0.6147	0.0047*	0.0030*	0.0030*
3 Days	0.0002*	0.0281*	0.4420*	0.7210*
5 Days	0.1050*	0.0104*	1.0000*	1.0000*
7 Days	0.4420*	0.0030*	0.9590*	0.9590*
14 Days	0.0104*	0.0002*	0.0104*	0.0104*
28 Days	0.1050*	0.2340*	0.1050*	0.1050*
42 Days	1.0000	0.0099	1.0000	1.0000

Table 5.2: T Test analysis comparing fibrin and collagen in control and treated cases.

	P Value							P Value							P Value						
	Peritoneal Tissue Generation							Uterine Horn Tissue Generation							Total Tissue Generation						
Injury Time	Basophilic Cells	Eosinophilic Cells	Inflammatory Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Inflammatory Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Inflammatory Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.
1 Hour	0.0012**	0.0365	0.0012*	0.5998	-	0.3422	0.0012*	0.0167*	0.8531	0.0066	0.6206	0.5170*	0.8330*	0.0171	0.0006*	0.0468	0.0006*	0.4560**	0.3830*	0.3830*	<0.0001
4 Hours	0.0019	0.0050	0.0009	0.8780*	0.1050*	0.2820	0.0015	0.0027	0.0271	0.0026	0.7100*	-	0.8050*	0.0026	<0.0001	0.0005	<0.0001	0.8780*	0.0379*	0.2993	<0.0001
8 Hours	0.0031*	0.3720	0.1211	0.8310	-	0.5639	0.1240	0.0424*	0.0324	0.0242**	0.9227	0.2300*	0.2032	0.0081	0.0011*	0.0283	0.0281**	0.9249	0.2340*	0.1300*	0.0082
16 Hours	0.0121*	0.0242**	0.0033	0.6179	0.0121*	0.2875	0.0047	0.0444*	0.0007	<0.0001	0.5416	0.2151	0.7110**	<0.0001	0.0002*	0.9590**	0.1050**	0.1950*	<0.0001	0.8646	0.6450*
1 Day	0.0016*	0.2957	0.0787	0.0045	0.0016*	0.9430	0.3027	0.0016*	0.6220*	0.0932	0.0451*	0.0016*	0.0929	0.2323	0.0002*	0.0268	0.0650**	0.0104*	0.0002*	0.4182	0.3280**
3 Days	0.0127*	0.1810*	0.5730*	0.3967	0.4825	0.0426*	0.6194	0.0667	0.3520*	0.4760*	0.1384	0.8902	0.2570*	0.2759	0.0030*	0.1050*	1.0000*	0.2785	1.0000*	0.0002*	0.7436
5 Days	0.0080*	0.1752	0.5730*	<0.0001	0.6142	0.6620*	0.2090	0.0293*	0.6620*	0.4910*	0.0354	0.6201	0.3450*	0.6620**	0.0006*	0.9590*	0.0499*	0.0920	0.8780**	0.4420*	0.9590**
7 Days	0.1210*	0.0059*	0.0721*	0.0721*	0.0190	0.1890*	0.3525	0.0728*	0.2528	0.8050*	0.0078	0.0157	0.0728*	0.3030	0.0019*	0.0155	0.1050*	0.2200	0.0004	0.0070*	0.0589
14 Days	0.7790*	0.0003**	0.0200*	0.0003*	0.5427	0.1649	0.2320*	0.8360*	0.3660*	0.9450*	0.0338	0.2203	0.6280*	0.8360*	0.6450*	0.0002**	0.0070*	0.0011*	0.3820**	0.9590*	0.9471
28 Days	0.7210*	0.0002*	0.0002*	0.0002**	0.3222	0.2340*	0.0296	-	0.3970*	0.3970*	0.0003**	0.0655	0.1890*	0.8448	0.4420*	0.0011*	0.0148*	<0.0001	0.0008	0.0019*	0.1045
42 Days	0.3360**	0.6130*	0.4630**	0.4630*	0.2959	0.7790*	0.3360**	0.4910*	0.2820*	0.1420*	0.0109	0.3411	0.4910*	0.4527	0.3820*	0.1791	0.2340*	0.0524	0.1197	0.1869	0.1583

Table 5.3: T test analysis of number densities with PTG, UTG and TTG for control and treated cases.

	P Value							P Value							P Value						
	Peritoneal Tissue Generation							Uterine Horn Tissue Generation							Total Tissue Generation						
Injury Time	Basophilic Cells	Eosinophilic Cells	Inflammatory Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Inflammatory Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.	Basophilic Cells	Eosinophilic Cells	Inflammatory Cells	Macrophages	Fibroblasts	Lymphocytes	Total No.
1 Hour	0.0012*	0.0047*	0.0012*	0.0614	-	0.3926	0.0012*	0.0146	0.7990	0.0068	0.4990	0.5170*	0.8330*	0.0167	0.0006*	0.0023*	0.0006*	0.0070*	0.3830*	0.3830*	0.0006*
4 Hours	0.0019**	0.0002**	0.0002*	0.0650*	0.1050*	0.5843	0.0002*	0.0012*	0.0047*	0.0012*	0.4450*	-	0.5340*	0.0012*	0.0002*	0.0002**	0.0002*	0.0650*	0.0379*	0.4420*	0.0002**
8 Hours	0.0031*	0.0932*	0.0295*	0.3038	-	0.6220*	0.0451	0.0095**	0.0667**	0.0095**	0.9140*	0.2570*	0.6172	0.0095**	0.0006*	0.0104**	0.0047**	0.2576	0.2340*	0.8455	0.0047**
16 Hours	0.0121*	0.0121**	0.0121*	0.0469	0.0121*	0.2875	0.0121*	0.0444*	0.0228	0.0011	0.9643	0.2282	0.8462	0.0067	0.0002*	0.0003**	0.0002**	0.0875	0.0773	0.1950**	0.0002**
1 Day	0.0016*	0.6048	0.2840**	0.0080	0.0057	0.8330*	0.6553	0.0016*	0.0557	0.3175	0.0243	0.0028	0.1710*	0.0421	0.0002*	0.0313	0.0051	0.0148*	0.0006*	0.2773	0.0429
3 Days	0.0127*	0.8520*	0.0080*	0.3450*	0.0112	0.0426*	0.0027**	0.0667*	0.3520*	0.4760*	0.2570*	1.0000**	0.2570*	1.0000**	0.0030*	0.2790*	0.0207*	0.0499**	0.0104**	0.0002*	0.0148**
5 Days	0.0080*	0.6583	0.1420*	0.7148	0.1810**	0.6620*	0.2820**	0.0080*	0.5730*	0.0027*	0.0027**	0.0007*	0.1810*	0.0007*	0.0002*	0.0830*	0.0379*	0.2208	0.0070**	0.2340*	0.0207**
7 Days	0.0939*	0.0541*	0.1520*	0.3825	0.0022**	0.0939*	0.0140*	0.0728*	0.5350**	0.1650*	0.3830*	0.0006*	0.0728*	0.0006**	0.0030*	0.1050*	0.3820*	0.9590*	0.0003*	0.0047*	0.0011*
14 Days	1.0000*	0.0058	0.0205*	0.0721*	0.0012*	0.7790*	0.0093*	0.8360*	0.3660*	0.6280*	0.8523	0.0223	0.5340*	0.0400	0.8780*	0.0038	0.0148*	0.3280*	0.0011*	0.6450*	0.0070*
28 Days	0.7210*	0.0003*	0.0003*	0.0207*	0.8780*	0.2340*	0.5050*	-	0.4630*	0.4630*	0.0401*	0.3360*	0.1890*	0.2062	0.4420*	0.0006*	0.0104*	0.0379*	0.0003*	0.0019*	0.0499*
42 Days	0.3360**	0.7790*	0.4630**	0.7640	0.0498	0.7790*	0.0340	0.2820*	0.2820*	0.0293*	0.0426**	0.0007*	0.4910*	0.0007*	0.1950*	0.2426	0.1610*	0.1164	0.0006*	0.3820*	0.0112

Table 5.4: T test analysis of number totals with PTG, UTG and TTG for control and treated cases.

	P Value						
	Platelets	RBC	WBC	Basophilic Cells	Lymphocytes	Monocytes	Eosinophilic Cells
Blood Sample 1	0.0091*	0.8190*	<0.0001**	0.3504	0.0433	0.1964	0.1053
Blood Sample 2	0.8016	0.0236*	<0.0001**	0.0986*	0.0050	0.8510*	0.1804
Blood Sample 3	0.7140	0.3920*	<0.0001**	0.0100*	<0.0001*	0.4860*	0.3350*

Table 5.5: T test analysis comparing full and differential blood counts for control and treated cases at blood samples 1, 2 and 3.
* = Normality test failed; Mann-Whitney Rank Sum test ran;
** = Equal variance test failed; Mann-Whitney Rank Sum test ran.

APPENDIX VI

Animal Number	Operative Time	Treatment/ Application Method	Fibrin Sealant Batch Number	Surgery & Necropsy Notes	General Adhesions	Animal Side	Number of Scrapes		Volume of Sealant Applied (ml)	Experimental Area Adhesions	? Fibrin clot remaining
							Uterine horn	Peritoneal wall			
FIB 13	40	Device Prepared Vivostat SPRAY	F96-11-01	LHS - u/horn adhered to itself where folded back (no sealant)	YES	LHS RHS	- -	- 26	0.66 0.52	NO NO	NO YES
FIB 14	32	"		LHS - cyst on serosal surface of u/horn	YES	LHS RHS	50 20SB 36	20 18SB 17	0.75 0.90	NO NO	YES NO
FIB 15	26	"	F96-11-02	skin suture leakage	YES	LHS RHS	40 33	40 21	0.58 0.46	NO NO	YES NO
FIB 16	26	"		-	YES	LHS RHS	30 45 10SB	20 43	0.67 0.93	NO one (cyst on adhesion)	YES YES
FIB 17	20	"	F96-11-03	-	YES	LHS RHS	14 20	13 16	0.61 0.40	YES (50%) NO	YES YES
FIB 18	25	"		-	YES	LHS RHS	22 29	22 17	0.52 0.70	NO NO	YES YES
FIB 19	25	Device Prepared Vivostat UNES GUN	F96-11-04	excess fat	YES	LHS RHS	16 19	17 10	1.50 1.00	YES (50%) one area	NO NO
FIB 20	20	"		RHS - wall perforated during srapping	YES	LHS RHS	8 10	24 7	1.25 1.25	one area one end	NO NO
FIB 21	26	"	F96-11-05	both sides left for 2 mins before treated	YES	LHS RHS	17 15	12 11	0.90 0.95	NO NO	YES YES
FIB 22	23	"		LHS u/horn perforated during scrapping	YES	LHS RHS	11 4	12 17	1.00 1.50	YES (100%) 2 areas	? YES
FIB 23	23	"	F96-11-06	both sides left for 5 mins before treated	YES	LHS RHS	7 8	13 8	0.50 0.60	NO YES (50%)	NO -
FIB 24	21	"	F96-11-07	RHS p/wall heavy bleed (vien involved)	YES	LHS RHS	10 12	12 12	1.00 1.20	NO NO	YES YES
FIB 25	22	Device Prepared Vivostat NEEDLE	F96-11-08	fat necrosis	YES	LHS RHS	- 7	- 9	0.77 0.88	NO NO	YES NO
FIB 26	19	"		fat involvement	YES	LHS RHS	6 16	9 10	0.64 1.20	NO NO	- YES
FIB 27	21	"	F96-11-09	fat involvement	YES	LHS RHS	7 7	10 9	0.76 0.80	NO YES	NO NO
FIB 28	21	"		large volume of fat necrosis	YES	LHS RHS	8 9	9 9	0.71 0.90	NO NO	- -
FIB 29	23	"	F96-11-10	exp. area close to p/suture line	YES	LHS RHS	10 10	19 13	0.91 0.85	NO NO	YES NO
FIB 30	17	"		small serosal cyst on p/suture line exp. area close to p/suture line	YES	LHS RHS	8 7	11 11	0.91 1.20	NO NO	NO YES
FIB 31	19	Bench Prepared Vivostat UNES GUN	F96-11-11	-	YES	LHS RHS	8 11	10 14	0.80 0.60	NO NO	YES YES
FIB 32	24			-	YES	LHS RHS	10 20	10 12	0.40 0.40	NO NO	YES YES
FIB 33	19		F96-11-12	small serosal cyst on p/suture line	YES	LHS RHS	12 15	15 8	1.00 0.60	NO NO	YES YES
FIB 34	21			small serosal cyst on p/suture line	YES	LHS RHS	12 11	13 9	1.00 1.00	NO NO	YES YES
FIB 35	20		F96-11-13	exp. area very close to p/suture line	YES	LHS RHS	12 9	12 8	1.40 0.60	NO NO	YES YES
FIB 36	18				YES	LHS RHS	10 15	13 16	0.80 1.00	NO NO	YES YES

Table 6.1: Macroscopic observations

Treatment	Percentage Incidence of PSAs	Percentage Incidence of Sealant Remaining
Controls	100.0%	N/A
Device Prepared Spray	16.7%	75%
Device Prepared UNES Gun	58.3%	41.7%
Device Prepared Needle	8.3%	33.3%
Bench Prepared UNES Gun	0%	100%

Table 6.2: Macroscopic results.

Treatment		Post Surgical Adhesion	Percentage of PSA	Fibrotic Tissue Generation				Damage Zone		Peritoneal Reaction Zone	Fibrin Sealant Remaining	Cell Concentration
				Peritoneal	Uterine Horn	Free Floating	Total	Peritoneal	Uterine Horn			
Control	**Mean**	**73.93**	**100.00**	**48.71**	**12.42**	**1.01**	**136.07**	**44.29**	**20.26**	**108.62**	**0.00**	**1.61**
	STD	45.18		13.13	18.17	1.80	64.61	20.99	18.69	28.42	0.00	2.54
	SEM	12.53		3.64	5.04	0.50	17.92	5.82	5.18	7.88	0.00	0.70
	CV%	16.95		7.48	40.59	49.45	13.17	13.14	25.58	7.26	#DIV/0!	43.73
Device Prepared Spray	**Mean**	**17.32**	**23.43**	**101.93**	**22.38**	**3.23**	**144.86**	**31.32**	**7.49**	**109.62**	**10.43**	**26.87**
	STD	24.18		40.03	16.44	4.50	52.27	23.11	9.51	44.64	11.71	20.50
	SEM	6.98		11.56	4.75	1.30	15.09	6.67	2.75	12.89	3.38	5.92
	CV%	40.30		11.34	21.21	40.31	10.42	21.30	36.68	11.76	32.40	22.03
Device Prepared Unes Gun	**Mean**	**39.37**	**53.25**	**101.27**	**22.12**	**1.54**	**164.31**	**33.73**	**12.87**	**118.49**	**6.87**	**23.80**
	STD	65.13		47.34	11.85	1.94	93.27	22.78	13.69	45.43	11.77	19.93
	SEM	18.80		13.67	3.42	0.56	26.93	6.58	3.95	13.11	3.40	5.75
	CV%	47.76		13.50	15.46	36.27	16.39	19.49	30.70	11.07	49.46	24.17
Device Prepared Needle	**Mean**	**24.66**	**33.36**	**96.31**	**17.17**	**3.85**	**141.99**	**35.46**	**11.93**	**91.78**	**9.40**	**19.63**
	STD	27.49		40.60	14.71	3.75	71.25	21.82	13.81	21.74	11.20	19.14
	SEM	7.94		11.72	4.25	1.08	20.57	6.30	3.99	6.27	3.23	5.53
	CV%	32.17		12.17	24.73	28.12	14.48	17.76	33.42	6.84	34.37	28.15
Bench Prepared Unes Gun	**Mean**	**5.40**	**7.31**	**80.46**	**24.84**	**5.18**	**115.88**	**25.32**	**11.72**	**88.64**	**56.93**	**51.67**
	STD	8.26		43.28	22.35	5.15	60.57	20.24	13.29	16.80	46.16	25.42
	SEM	2.38		12.49	6.45	1.49	17.49	5.84	3.84	4.85	13.32	7.34
	CV%	44.12		15.53	25.98	28.68	15.09	23.08	32.71	5.47	23.40	14.20

Table 6.3: Tissue zone volumes (mm^3) for each treatment group.

	P VALUES								
Treatment	Post Surgical Adhesion	Fibrotic Tissue Generation				Damage Zone		Peritoneal Reaction Zone	Cell Concentration
		Peritoneal	Uterine Horn	Free Floating	Total	Peritoneal	Uterine Horn		
Device Prepared Spray	0.0008	0.0001	0.0535	0.209	0.7133	0.121	0.0208	0.9465	<0.0001
Unes Gun	0.018	0.00146	0.024	0.495	0.479	0.121	0.221	0.5174	<0.0001
Needle	0.0035	0.00146	0.0727	0.0165	0.8295	0.3135	0.341	0.112	0.00192
Bench Prepared Unes Gun	<0.0001	0.0606	0.149	0.0153	0.4296	0.024	0.121	0.109	<0.0001

Table 6.4: T Test Analysis of Treatment Groups Compared to Control.

P VALUES									
Post Surgical Adhesion	Fibrotic Tissue Generation				Damage Zone		Peritoneal Reaction Zone	Fibrin Sealant Remaining	Cell Concentration
	Peritoneal	Uterine Horn	Free Floating	Total	Peritoneal	Uterine Horn			
0.0737	0.585	0.726	0.206	0.429	0.47	0.51	0.122	0.0014*	0.0028*

* = Statistical significant difference exists between Bench Prepared Unes Gun and Device Prepared Applications

Table 6.5: One way ANOVA analysis of treatment groups.

		P VALUES			
		Device Prepared			Bench Prepared
		Spray	UNES Gun	Needle	UNES Gun
Device Prepared	**Spray**	-	0.0022**	0.0023	0.1750**
"	**UNES Gun**	-	-	0.0496	0.5160
"	**Needle**	-	-	-	0.5440**
Bench Prepared	**UNES Gun**	-	-	-	-

Table 6.6: T test Analysis comparing volume of fibrin sealant applied for each treatment group.
** = Equal Variance Test Failed; Mann-Whitney Rank Sum Test Ran.

		P VALUES			
		Device Prepared			Bench Prepared
		Spray	UNES Gun	Needle	UNES Gun
Device Prepared	**Spray**	-	0.6185	0.1000*	0.1373
"	**UNES Gun**	-	-	0.4798	0.1749
"	**Needle**	-	-	-	0.3213
Bench Prepared	**UNES Gun**	-	-	-	-

Table 6.7: T test analysis comparing fibrin I concentration (mg/ml) for each treatment group.
* = Normality Test Failed; Mann-Whitney Rank Sum Test Ran

APPENDIX VII

CRYO Preparation Protocol

1. Thaw 2 litres of plasma.
2. Filter through a damp cotton cloth.
3. Add 4 units/ml heparin.
4. Aliquot into 45ml volumes.
5. Add 4.5ml of cooled ethanol to each aliquot.
6. Precipitate on ice for 30 minutes.
7. Centrifuge at 3000rpm at 5°C for 10 minutes.
8. Collect concentrate and pool.
9. Re-spin pooled fraction at 3000rpm at 5°C for 5 minutes.
10. Combine precipitates and heat to 37°C or until in solution.
11. Assay the concentration of the pooled fraction.
12. Thaw plasma required to produce a resulting combination of pooled fraction and normal plasma at 6mg/ml (the concentration which is often used in device studies).
13. Treated thawed normal plasma as in steps 2 and 3 above before addition of concentrated pooled fraction.
14. Assay spiked plasma for fibrinogen concentration.

Animal Number	Operative Time	Treatment/ Application Method	Surgery & Necropsy Notes	General Adhesions	Animal Side	Number of Scrapes		Volume of Sealant Applied (ml)	Experimental Area Adhesions	? Fibrin clot remaining
						Uterine horn	Peritoneal wall			
FIB 37	27	Controls	multiple bladder & omental adhesions	YES	LHS	12	9	N/A	YES (100%)	N/A
					RHS	12	9	"	"	"
FIB 38	25	"	bladder adhesions only; cyst on RHS u/horn	YES	LHS	6	9	"	"	"
					RHS	5	9	"	"	"
FIB 39	18	"	bladder & omental adhesions;	YES	LHS	5	9	"	"	"
			op. site far away from p/suture line		RHS	5	9	"	"	"
FIB 40	21	"	small haematoma on p/suture line	YES	LHS	6	9	"	"	"
			severe bladder & omental adhesions		RHS	6	9	"	"	"
FIB 41	22	"	small serosal cyst on p/suture linemultiple bladder & omental adhesions	YES	LHS	6	9	"	"	"
			RHS inflammed with severe adhesions		RHS	6	9	"	"	"
FIB 42	18	"	bladder adhesions only	YES	LHS	6	9	"	"	"
			RHS p/wall slightly inflammed; RHS: severe adhesions		RHS	6	9	"	"	"
FIB 43	25	Tissucol®	bladder adhesions only	YES	LHS	6	9	0.50	YES (55%)	YES
		4 Units Thrombin	both u/horn inflammed; RHS - tissue reaction		RHS	6	9	0.50	YES	YES
FIB 44	19	"	minor bladder & omental adhesions	YES	LHS	5	9	0.40	YES (65%)	-
					RHS	6	9	0.00	YES (50%)	-
FIB 45	40	Novel Fibrin	sides left before treated	YES	LHS	6	9	0.52	NO	NO
		Sealant	fat & omental adhesions		RHS	6	9	0.76	NO	NO
FIB 46	20	"	not good delivery	YES	LHS	6	9	0.88	NO	NO
			fat and caecal adhesions		RHS	6	9	0.90	NO	NO
FIB 47	26		necrotic bladder & ascites		LHS	6	9	0.82	NO	NO
			slight bladder & omental adhesions		RHS	6	9	0.99	NO	NO
FIB 48	24	"	bladder adhesions only	YES	LHS	6	9	0.82	NO	NO
			exp.site close to p/suture line		RHS	6	9	1.30	NO	NO
FIB 49	24		bladder adhesions only		LHS	6	9	0.90	NO	YES
			LHS close to p/suture line		RHS	6	9	1.11	?	YES
FIB 50	25		lots of ascites fluid; extensive bladder adhesions		LHS	6	10	0.94	NO	YES
			RHS bowel inflammed; LHS u/horn inflammed		RHS	6	9	1.18	NO	YES
FIB 51	22	Cryo	fat adhesions	YES	LHS	8	9	0.40	NO	? NO
		4 Units Thrombin			RHS	8	9	0.50	NO	YES
FIB 52	20	Cryo	fat and omental adhesions	YES	LHS	10	9	0.45	NO	?
		4 Units Thrombin			RHS	10	9	0.55	YES	?
FIB 53	26		LHS bleeder		LHS	8	9	0.60	NO	?
			severe fat & omental adhesions		RHS	6	9	0.55	NO	?
FIB 64	30	Tissucol®	8 mins btwn scrape & treatment	YES	LHS	8	6	0.40	NO	YES
		500 UnitsThrombin	omental adhesions		RHS	8	6	0.25	NO	YES
FIB 65	18	"	u/horns severe wounds; friable tissue	YES	LHS	8	6	0.35	YES	YES
			omental adhesions; both u/horn inflammed		RHS	8	6	0.45	NO	YES
FIB 66	19		quite severe omental adhesions		LHS	8	6	0.25	YES	-
			both u/horns inflammed		RHS	8	8	0.30	YES	YES
FIB 67	19	Tissucol®	u/horns bleeders	YES	LHS	8	6	0.43	YES	-
		4 UnitsThrombin	both horns very inflammed		RHS	8	8	0.52	YES	-
FIB 68	21	Cryo	bladder & omental adhesions	YES	LHS	8	8	1.20	YES	YES
		500 Units Thrombin	v.v. large horns		RHS	8	8	1.00	YES	YES
FIB 69	21	"	good application	YES	LHS	8	8	0.80	NO	YES
			bladder & fat adhesions; horns inflammed		RHS	9	8	1.30	NO	YES
FIB 70	21	"	cryo btwn p/suture line & skin	YES	LHS	8	10	0.90	YES	YES
			large reaction on p/suture line; many skin adhesions; horns inflammed	RHS	8	12	1.5	YES	YES	

Table 7.1: Macroscopic observations

Treatment		Post Surgical Adhesion	Percentage PSAs		Fibrotic Tissue Generation				Damage Zone		Peritoneal Reaction Zone	Fibrin Sealant Remaining	Cell Conc*
			Incidence	Reduction	Peritoneal	Uterine Horn	Free Floating	Total	Peritoneal	Uterine Horn			
Control	**Mean**	**73.93**	**100.00**	**N/A**	**48.71**	**12.42**	**1.01**	**136.07**	**44.29**	**20.26**	**108.62**	**0.00**	**1.61**
	STD	45.18	-	-	13.13	18.17	1.80	64.61	20.99	18.69	28.42	0.00	2.54
	SEM	12.53	-	-	3.64	5.04	0.50	17.92	5.82	5.18	7.88	0.00	0.70
	CV%	16.95	-	-	7.48	40.59	49.45	13.17	13.14	25.58	7.26	#DIV/0!	43.73
Vivostat	**Mean**	**11.30**	**15.28**	**84.72**	**68.75**	**6.69**	**2.37**	**89.11**	**18.02**	**2.50**	**79.58**	**21.20**	**29.62**
(Needle)	STD	11.32	-	-	37.93	6.93	2.37	41.10	16.40	3.19	19.83	26.06	24.74
	SEM	3.27	-	-	10.95	2.00	0.68	11.87	4.73	0.92	5.72	7.52	7.14
	CV%	28.91	-	-	15.93	29.92	28.90	13.32	26.27	36.84	7.19	35.48	24.11
CRYO	**Mean**	**21.06**	**28.49**	**71.51**	**111.20**	**13.95**	**1.49**	**147.70**	**48.78**	**8.89**	**88.18**	**13.64**	**22.87**
4 Units	STD	21.45	-	-	26.43	15.35	1.13	39.36	24.06	8.51	26.93	11.51	18.85
Thrombin	SEM	8.76	-	-	10.79	6.27	0.46	16.07	9.82	3.47	11.00	4.70	7.70
	CV%	41.58	-	-	9.70	44.91	31.09	10.88	20.13	39.08	12.47	34.46	33.66
CRYO	**Mean**	**179.98**	**243.46**	**-143.46**	**136.24**	**33.95**	**0.76**	**350.94**	**46.13**	**37.38**	**174.90**	**97.07**	**79.16**
500 Units	STD	185.72	-	-	53.23	23.78	0.95	231.12	14.06	45.46	14.30	51.20	27.44
Thrombin	SEM	75.82	-	-	21.73	9.71	0.39	94.36	5.74	18.56	5.84	20.90	11.20
	CV%	42.13	-	-	15.95	28.59	50.61	26.89	12.44	49.65	3.34	21.53	14.15
Tissucol	**Mean**	**106.12**	**143.55**	**-43.55**	**82.34**	**14.45**	**1.32**	**204.23**	**50.62**	**18.08**	**122.09**	**42.47**	**35.33**
4 Units	STD	107.71	-	-	56.61	12.12	1.52	169.56	19.82	6.31	38.22	46.20	36.97
Thrombin	SEM	48.17	-	-	25.32	5.42	0.68	75.83	8.86	2.82	17.09	20.66	16.54
	CV%	45.39	-	-	30.75	37.53	51.57	37.13	17.51	15.62	14.00	48.65	46.81
Tissucol	**Mean**	**69.42**	**93.90**	**6.10**	**137.09**	**28.26**	**3.27**	**238.04**	**35.20**	**2.71**	**137.72**	**110.70**	**72.48**
500 Units	STD	91.45	-	-	40.86	15.59	2.61	99.89	15.86	3.66	43.35	63.47	27.03
Thrombin	SEM	37.33	-	-	16.68	6.36	1.06	40.78	6.47	1.49	17.70	25.91	11.04
	CV%	53.78	-	-	12.17	22.52	32.57	17.13	18.39	55.15	12.85	23.41	15.23

Table 7.2: Quantitative volumetric results.

	P VALUE									
	Post Surgical Adhesion	Fibrotic Tissue Generation				Damage Zone		Peritoneal Reaction Zone	Fibrin Sealant Remaining	Cell Conc^{n}
		Peritoneal	Uterine Horn	Free Floating	Total	Peritoneal	Uterine Horn			
Controls vs Vivostat (Needle)	<0.0001**	0.3840**	0.9350*	0.1080*	0.0426	0.0021	<0.0001**	0.0074	N/A	<0.0001*
Controls vs CRYO 4 Units Thrombin	0.0152	<0.0001	0.630*	0.2350*	0.6911	0.6838	0.1722	0.1574	N/A	0.0014*
Controls vs CRYO 500 Units Thrombin	0.3130**	<0.0001*	0.0316*	0.5670*	0.0033*	0.8490	0.8260*	0.0010**	N/A	<0.0001*
Controls vs Tissucol 4 Units Thrombin	0.3717	0.3240**	0.2370*	0.5850*	0.2205	0.5694	0.8041	0.4233	N/A	0.0016*
Controls vs Tissucol 500 Units Thrombin	0.8854	<0.0001*	0.0484*	0.0429*	0.0154	0.3607	0.0159**	0.0964	N/A	<0.0001*
CRYO 4 Units Thrombin vs 500 Units Thrombin	0.0640	0.3624	0.1141	0.2582	0.0597	0.8203	0.1623	<0.0001	0.0030	0.0020
Tissucol 4 Units Thrombin vs 500 Units Thrombin	0.5555	0.0951	0.1414	0.1764	0.6896	0.1846	0.0007	0.5461	0.0770	0.3605

Table 7.3: T Test analysis between controls and treated groups.
* = Normality Test Failed; Mann-Whitney Rank Sum Test Ran.
** = Equal Variance Test Failed; Mann-Whitney Rank Sum Test Ran.

One Way Anova, with multiple comparison via Dunn's Method showed significant differences ($P<0.05$) between fibrin sealant remaining for:

Vivostat vs CRYO 500 Units Thrombin

Vivostat vs Tissucol 500 Units Thrombin.

APPENDIX VIII

Treatment	Injury Site		Volume of Post Surgical Adhesion (mm^3)	Percentage Post Surgical Adhesion		Volume of Inflammation (mm^3)	Volume of Fibrin Sealant Remaining (mm^3)
				Incidence	Reduction		
Control	**Colon**	**MEAN**	**207.25**	**100.00**	**0.00**	**45.54**	**N/A**
		SD	157.01	-	-	56.97	N/A
		SEM	78.40	-	-	28.48	N/A
"	**Caecum**	**MEAN**	**31.33**	**100.00**	**0.00**	**0.00**	**N/A**
		SD	10.19	-	-	0.00	N/A
		SEM	7.21	-	-	0.00	N/A
"	**Stomach**	**MEAN**	**248.11**	**100.00**	**0.00**	**9.01**	**N/A**
		SD	36.29	-	-	8.42	N/A
		SEM	20.95	-	-	4.86	N/A
	Overall	**MEAN**	**181.78**	**100.00**	**0.00**	**23.24**	**N/A**
		SD	131.22	-	-	41.16	N/A
		SEM	43.74	-	-	13.72	N/A
Human Fibrin Sealant	**Colon**	**MEAN**	**42.83**	**20.67**	**79.33**	**280.20**	**128.87**
		SD	21.39	-	-	84.82	50.11
		SEM	10.69	-	-	42.41	25.06
"	**Caecum**	**MEAN**	**8.96**	**28.60**	**71.40**	**375.02**	**162.82**
		SD	12.67	-	-	4.02	39.92
		SEM	8.96	-	-	2.84	28.23
"	**Stomach**	**MEAN**	**204.47**	**82.41**	**17.59**	**429.27**	**174.12**
		SD	159.23	-	-	259.56	14.22
		SEM	112.59	-	-	183.54	10.05
	Overall	**MEAN**	**74.77**	**41.10**	**58.90**	**341.17**	**148.67**
		SD	102.31	-	-	131.83	42.42
		SEM	36.17	-	-	46.61	15.00
Porcine Fibrin Sealant	**Colon**	**MEAN**	**0.00**	**0.00**	**100.00**	**9.17**	**100.45**
		SD	0.00	-	-	9.72	16.75
		SEM	0.00	-	-	6.88	11.84
"	**Caecum**	**MEAN**	**0.00**	**0.00**	**100.00**	**16.28**	**132.38**
		SD	0.00	-	-	23.02	56.47
		SEM	0.00	-	-	16.28	39.93
"	**Stomach**	**MEAN**	**6.35**	**2.56**	**97.44**	**55.24**	**139.23**
		SD	0.00	-	-	0.00	0.00
		SEM	0.00	-	-	0.00	0.00
	Overall	**MEAN**	**1.27**	**0.70**	**99.30**	**21.23**	**120.98**
		SD	2.84	-	-	23.03	35.02
		SEM	1.27	-	-	10.30	15.66

Table 8.1: Quantitative results for each experimental site.

APPENDIX IX

Animal No: (PPSA)	Injury Site		Post Surgical Adhesion	% Post Surgical Adhesions		Tissue Generation			Damage		Inflamm-ation	Novel Fibrin Sealant
				Incidence	Reduction	Peritoneal	Colon/ Stomach	Total	Peritoneal	Colon/ Stomach		
Control Group												
10	**Colon**		689.06			234.67	83.79	1007.52	56.84	14.36	3.52	N/A
11			814.45			93.02	45.04	952.51	20.87	26.73	5.13	N/A
12			937.89			0.00	0.00	937.89	36.91	17.09	4.10	N/A
13			583.98			0.00	0.00	583.98	19.04	14.16	5.37	N/A
17			342.11			0.00	0.00	342.11	23.41	11.71	0.00	N/A
		Mean	**673.50**	**100.00**	**0.00**	**65.54**	**25.77**	**764.80**	**31.42**	**16.81**	**3.62**	N/A
		STD	227.95	-	-	102.77	37.85	289.60	15.85	5.87	2.16	N/A
		SEM	101.94	-	-	45.96	16.93	129.51	7.09	2.62	0.97	N/A
		%CV	15.14	-	-	70.13	65.69	16.93	22.56	15.61	26.68	N/A
10	**Stomach**		1064.08			128.28	38.69	1231.05	61.90	21.58	6.92	N/A
13			1400.94			0.00	58.86	1459.80	13.33	28.62	10.73	N/A
14			627.54			48.14	0.00	675.68	24.07	37.35	5.40	N/A
16			1345.01			0.00	0.00	1345.01	37.40	32.84	1.95	N/A
20	"		675.00			0.00	0.00	675.00	34.28	11.72	0.59	N/A
	"	**Mean**	**1022.51**	**100.00**	**0.00**	**35.28**	**19.51**	**1077.31**	**34.20**	**26.42**	**5.12**	N/A
		STD	362.53	-	-	56.01	27.65	375.75	18.13	10.06	4.04	N/A
		SEM	162.13	-	-	25.05	12.37	168.04	8.11	4.50	1.81	N/A
		%CV	15.86	-	-	70.99	63.38	15.60	23.72	17.03	35.34	N/A
Novel Fibrin Sealant Group												
14	**Colon**		0.00			186.04	219.73	405.76	3.66	19.78	127.44	9.89
16			0.00			374.70	171.09	545.79	23.95	2.99	90.68	0.00
18			0.00			197.86	67.92	265.78	12.92	7.01	134.37	0.00
19			0.00			224.30	101.84	326.13	14.18	6.12	148.24	0.00
20			0.00			109.85	49.82	159.67	12.13	2.87	24.27	0.00
21			77.50			239.77	130.78	448.05	18.47	14.23	231.29	31.18
		Mean	12.92	1.92	98.08	222.08	123.53	358.53	14.22	8.83	126.05	6.84
		STD	31.64	-	-	87.28	64.13	137.48	6.79	6.77	68.19	12.56
		SEM	12.92	-	-	35.63	26.18	56.13	2.77	2.76	27.84	5.13
		%CV	100.00	-	-	16.04	21.20	15.65	19.48	31.28	22.09	74.92
11	**Stomach**		982.08			0.00	0.00	982.08	6.42	25.24	855.47	107.79
12			720.63			75.45	41.63	837.70	25.69	13.98	299.18	5.85
17			199.32			0.00	0.00	199.32	4.13	6.67	96.48	48.24
18			297.50			471.48	33.20	802.19	18.93	4.98	276.25	32.87
19			34.28			455.76	214.55	704.59	16.50	17.14	302.15	6.67
21			87.02			526.55	213.70	827.27	22.86	54.25	295.22	18.18
		Mean	386.80	37.83	62.20	254.87	83.85	725.52	15.75	20.38	354.13	36.60
		STD	380.28	-	-	254.24	102.33	272.75	8.74	18.15	258.06	38.47
		SEM	155.25	-	-	103.79	41.77	111.35	3.57	7.41	105.35	15.70
		%CV	40.14	-	-	40.72	49.82	15.35	22.66	36.37	29.75	42.91

Table 9.1: Mean volumes (mm^3) for each tissue zone for control and novel fibrin sealant groups, together with SDs, SEMs and %CV.

	P VALUES						
Injury Site	Post Surgical Adhesion	Tissue Generation			Damage		Inflammation
		Peritoneal	Colon/ Stomach	Total	Peritoneal	Colon/ Stomach	
Colon	0.0043	0.023	0.0153	0.0134	0.0383	0.0691	0.0043
Stomach	0.0201	0.247	0.2087	0.1053	0.0538	0.525	0.0043
Overall Mean	0.0017	0.0067	0.0093	0.0113	0.0041	0.1998	<0.001

Table 9.2: T test analysis comparing control and novel fibrin sealant groups for volumes of each tissue zones.

	P VALUES							
Injury Site	Post Surgical Adhesion	Tissue Generation			Damage		Inflammation	Fibrin Sealant Remaining
		Peritoneal	Colon/ Stomach	Total	Peritoneal	Colon/ Stomach		
Controls	0.1059	0.8410*	0.8410*	0.2220*	0.8029	0.1022	0.4869	N/A
Treated	0.0043	1.0000	0.4396	0.0147	0.7413	0.1752	0.026	0.1019

Table 9.3: T test analysis comparing colon and stomach experimental sites of control and novel fibrin sealant groups for volumes of each tissue zones.
* = Normality test failed; Mann-Whitney Rank sum test ran.

APPENDIX X

Variable	Authors
Animal Species	
Rat	Chandy & Rhoads, 1946; Grosfeld *et al.*, 1973; Lindenberg & Lauritsen, 1984; de Virgilio *et al.*, 1990; Dulchavsky *et al*, 1991; Bothin & Hallberg, 1992; Evans *et al.*, 1993; Sheppard *et al.*, 1993; Sahin & Saglam, 1994; Alponat *et al.*, 1997.
Rabbit	Neuwirth and Khalaf, 1975; Nishimura *et al.*, 1983; Diamond *et al.*, 1987; De Laco *et al.*, 1994; Takeuchi *et al.*, 1997; Marana *et al.*, 1997; Rodgers *et al.*, 1997; De Laco *et al.*, 1998.
Pig	Fowler *et al.*, 1994; Christoforoni *et al.*, 1996; Reissman *et al.*, 1996.
Dog	Replogle *et al.*, 1966; Stevens, 1968; Youmons *et al*, 1968; Buckman *et al.*, 1975; Vander Salm *et al.*, 1986; Shimanuki *et al.*, 1987; Duncan *et al.*, 1988; McCabe Fowler *et al.*, 1991; Mitchell *et al.*, 1994.
Monkey	Larsson *et al.*, 1986; Kapur *et al.*, 1969; Liao *et al.*, 1973.
Cow (calf)	Baker *et al.*, 1989.
Chicken	Burns *et al.*, 1997.
Human	Adhesion Study Group, 1983; Krinsky *et al.*, 1984; Jansen, 1985; Rein & Hill, 1989; Jansen, 1985; Larsson *et al.*, 1986; Kram *et al.*, 1988; Malinak, 1990; Longaker *et al.*, 1991; Beck, 1997.
Induction Methods	
Rubbing of caecal surface	Conolly & Stephens FO, 1968; Milligan & Raftery, 1974.
Chemical trauma	Chandy & Rhoads, 1946.
Crushing caecal wall	Chandy & Rhoads, 1946.
Mesentric division	Ellis, 1962.
Polythene/ omental wrap around devascularised intestine	Ellis, 1962.
Liver lobe scraping with scalpel blade	Milligan & Raftery, 1974.
Severity of Techniques	
Rubbing	Conolly & Stephens FO, 1968; Milligan & Raftery, 1974.
Crushing	Chandy & Rhoads, 1946.
Scalpel scraping	Milligan & Raftery, 1974.
Site of PSA Induction	
Caecum	Chandy & Rhoads, 1946; Conolly & Stephens FO, 1968; Milligan & Raftery, 1974.
Intestine	Ellis, 1962.
Liver	Milligan & Raftery, 1974.
Uterine horn	Neuwirth and Khalaf, 1975; Nishimura *et al.*, 1983; Diamond *et al.*, 1987; De Laco *et al.*, 1994; Takeuchi *et al.*, 1997; Marana *et al.*, 1997; Rodgers *et al.*, 1997; De Laco *et al.*, 1998.
End Point	
Incidence	Ellis, 1962.
Length of adhesion attachment	O'Leary & Coakley, 1992.
Type of adhesion	Shimanuki *et al.*, 1987.
Macromorphological adhesion grading	Dargenio et al., 1986; Sheppard et al., 1993.

Table 10.1: PSA experimental model variations.

	PSA Volume (mm3)	% PSA		Fibrin Sealant Associated Reaction Volume (mm3)	Fibrin Sealant Remaining Volume (mm3)
		Incidence	Reduction		
Control Cases					
Mean	**89.91**	**100.00**	**-**	**-**	**-**
SD	52.11			-	-
SEM	18.42			-	-
CV%	20.49			-	-
Treated Cases					
Mean	**17.82**	**19.82**	**80.18**	**2.75**	**0.97**
SD	23.76			3.06	1.05
SEM	8.40			1.08	0.37
CV%	47.16			39.34	38.33

Table 10.2: Rat caecal abrasion study quantitative results.

Wet tissue (g^{-1})		
Protein mg	Activity CTA U	Tissue
16.9	412.0	Pancreas
32.3	70.0	Ovary
53.8	69.9	Oesophagus
58.6	69.2	Vena cava
40.5	67.6	Pleura
10.8	63.8	Testes
37.5	63.0	Bladder
38.8	60.3	Uterus
26.9	56.0	Duodenum
6.4	55.7	Liver
27.9	49.3	Prostate
26.7	49.1	Colon
48.0	49.0	Aorta
21.6	43.2	Adrenal
21.7	41.2	Pituitary
34.6	33.5	Lung
32.2	31.1	Gall-bladder
44.7	27.6	Ureter
3.2	22.7	Cerebrum
51.4	21.4	Thyroid
28.3	21.0	Peritoneum
2.2	19.4	Cerebellum
21.5	18.3	Heart
14.2	16.3	Omentum
26.7	13.9	Trachea
15.3	13.3	Breast
16.1	10.8	Kidney
9.7	9.7	Muscle
18.3	7.5	Stomach
25.9	<5.4	Spleen
21.6	0.0	Placenta

Dry tissue (g^{-1})		
Tissue	Activity CTA U	Protein mg
Pancreas	956.0	39.1
Testes	356.4	60.3
Ovary	303.0	139.8
Oesophagus	284.1	218.7
Uterus	266.8	171.7
Pituitary	265.8	140.0
Duodenum	235.3	113.0
Bladder	198.1	117.6
Liver	197.5	22.7
Vena cava	194.9	165.1
Prostate	181.2	102.6
Lung	176.3	182.1
Colon	147.9	80.4
Aorta	142.0	139.1
Gall-bladder	116.5	120.6
Cerebrum	115.2	16.2
Pleura	112.1	67.2
Cerebellum	91.9	10.4
Heart	78.9	92.7
Ureter	74.8	121.1
Adrenal	71.9	35.9
Thyroid	71.8	172.5
Kidney	43.7	65.2
Trachea	42.6	81.9
Peritoneum	40.0	53.9
Muscle	36.5	36.5
Stomach	30.0	73.2
Omentum	19.5	16.9
Breast	17.1	19.6
Spleen	<25.1	120.5
Placenta	0.0	108.5

Table 10.3: Comparison of the extractable fibrinolytic activity and protein per gram wet and dry weight of tissue, based on the 40 000 xg tissue extract supernatants (taken directly from Marshall, 1985).

Legend:

Protein and activity values per gram of dry tissue were calculated from the wet weight values by correcting for the water content estimated for each tissue (Section 6.2.1.2.). Generally, tissues with a large water content have a higher relative fibrinolytic activity ranking when expressed per gram dry weight compared with their position on a wet weight basis, the converse being true for tissues of low water content.

Injury Time	Animal No:	Length Density (x10-5/um2)		Length (x10-5um)	
		Actual	Mean	Actual	Mean
Control Cases					
16 Hours	105	0	0	0	0
	107	0		0	
1 Day	97	5.096	3.716	435.87	290.29
	99	2.335		144.71	
3 Days	101	7.59	9.635	478.33	1002.9
	103	11.68		1527.46	
5 Days	112	12.1	9.553	2286.65	2016.4
	115	7.006		1746.15	
7 Days	123	3.503	3.854	796.08	641.58
	125	4.204		487.08	
14 Days	117	4.671	6.54	2523.56	1822.6
	126	8.408		1121.65	
28 Days	119	5.839	5.372	448.54	1683.21
	121	4.904		2917.88	
42 Days	109	7.643	6.391	998.76	1193.82
	114	5.138		1388.87	

Injury Time	Animal No:	Length Density (x10-5/um2)		Length (x10-5um)	
		Actual	Mean	Actual	Mean
Treated Cases					
16 Hours	170	0	0	0	0
	172	0		0	
1 Day	150	0.876	0.438	45	22.5
	152	0		0	
3 Days	154	3.503	6.131	77.89	174.47
	156	8.758		271.05	
5 Days	138	12.84	12.725	296.55	897.15
	140	12.61		1497.75	
7 Days	142	7.785	9.898	543.11	599.11
	144	12.01		655.12	
14 Days	131	4.204	6.306	304.22	361.49
	134	8.408		418.76	
28 Days	146	5.255	6.715	302.81	312.3
	148	8.174		321.8	
42 Days	130	7.006	5.255	143.09	186.04
	135	3.503		228.986	

Table 10.4: Length density and total length of vessels within PSAs and tissue generation for control and novel fibrin sealant treated cases.

Time Point	Animal Number		Volume ($cm^{2)}$				% Difference
			Wax	Resin	Wax	Mean	
Control Cases							
4 Hours	FIB 87 LHS	PTG	8.00	12.00	-	10.00	20.00
8 Hours	FIB 93 RHS	U.Horn width	15.00	17.00	-	16.00	6.25
		U.Horn height	7.00	10.00	-	8.50	17.65
		Peritoneum height	5.00	6.00	-	5.50	9.09
		PSA	7.50	14.50	-	11.00	31.82
		PTG	1.50	5.50	-	3.50	57.14
		PSA	0.50	4.50	-	2.50	80.00
16 Hours	FIB 106 RHS	U.Horn width	16.00	17.00	-	16.50	3.03
		U.Horn height	16.00	15.00	-	15.50	3.23
		PSA	21.00	23.00	-	22.00	4.55
1 Day	FIB 99 RHS	U.Horn width	13.00	13.00	15.00	13.67	6.50
		U.Horn height	8.00	9.00	7.00	8.00	0.00
		Peritoneum height	7.00	8.00	6.00	7.00	9.52
		PSA	6.50	6.50	8.00	7.00	9.52
3 Days	FIB 101 LHS	U.Horn width	21.00	19.00	21.00	20.33	4.37
		PSA	2.50	15.00	12.00	9.83	49.72
5 Days	FIB 112 RHS	U.Horn height	8.00	8.00	9.00	8.33	5.33
		PSA	22.00	13.00	16.00	17.00	19.61
7 Days	FIB 129 RHS	U.Horn width	6.00	6.00	7.00	6.33	7.02
		U.Horn height	6.00	6.00	6.00	6.00	0.00
		Peritoneum height	6.00	7.00	6.00	6.33	7.02
		PSA	44.00	53.00	48.00	48.33	5.98
14 Days	FIB 119 LHS	U.Horn width	16.00	16.00	20.00	17.33	10.26
		U.Horn height	16.00	18.00	15.00	16.33	6.80
		Peritoneum height	4.00	5.00	5.00	4.67	9.52
		PSA	5.00	8.00	8.00	7.00	19.05
42 Days	FIB 116 LHS	PSA	29.00	38.00	49.00	38.67	17.82
Treated Cases							
I Hour	FIB 166 LHS	U.Horn width	9.00	10.00	8.00	9.00	7.41
		U.Horn height	8.00	9.00	8.00	8.33	5.33
		Peritoneum height	8.00	8.00	8.00	8.00	0.00
		Fibrin Sealant	21.00	35.00	19.00	25.00	26.67
		PTG	3.00	4.00	3.00	3.33	13.33
16 Hours	FIB 171 RHS	U.Horn width	9.00	13.00	11.00	11.00	12.12
		U.Horn height	10.00	10.00	8.00	9.33	9.52
		Peritoneum height	8.00	9.00	8.00	8.33	5.33
		Fibrin Sealant	16.00	28.00	20.00	21.33	20.83
		PTG	5.00	16.00	7.00	9.33	47.62
7 Days	FIB 144 LHS	U.Horn width	15.00	15.00	-	15.00	0.00
		Peritoneum height	10.00	9.00	-	9.50	5.26
		Fibrin Sealant	13.00	16.00	-	14.50	10.34
		PTG	4.00	4.00	-	4.00	0.00
42 Days	FIB 132 LHS	U.Horn width	11.00	13.00	12.00	12.00	5.56
		Peritoneum height	7.00	5.00	5.00	5.67	15.69
		PTG	7.00	5.00	4.00	5.33	20.83
	Mean						14.24
	TG Mean						27.40

Table 10.5: Comparison of tissue zone volumes for wax and resin processed sections.